Adaptation strategies of soil biodiversity (earthworms) to pesticides : mechanisms in play and ecosystemic cost assessment

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Stratégies d'adaptations de la biodiversité du sol à la pollution environnementale résiduelle établie en paysage agricole

Thèse soutenue à Odense (Danemark) le 19/03/2014

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Potentiel d'adaptation des lombricidés aux pollutions environnementales résiduelles établies en paysage agricole: mécanismes en jeu et coûts à l'écosystème

Résumé de thèse de doctorat en Français

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Ce mémoire de thèse est écrit en anglais et comporte, outre une introduction de 80 pages, trois articles scientifiques formant le corps du manuscrit. Ce résumé reprend et résume en français les principales questions et résultats de ces trois chapitres.

Introduction

Dans l'agriculture moderne, les champs cultivés sont des écosystèmes profondément modifiés par l'homme dans lesquels de hauts rendements sont obtenus grâce à des modes de gestion conventionnels à travers l'utilisation importante de pesticides, fertilisants et travail mécanique du sol. L'application de multiples pesticides a induit une contamination chronique des sols et de leur biota, en particulier les vers de terre (Lee 1985; Redondo et al. 1994; Luchini et al. 2000; Gevao et al. 2001). La rémanence de nombreux pesticides, tels que l'herbicide atrazine (Solomon et al. 1996; Giddings et al. 2005), pose des problèmes environnementaux majeurs en ce qui concerne la contamination résiduelle des sols agricoles, mais aussi leur potentiel de transfert vers les eaux superficielles et souterraines, et enfin leur effets nocifs sur la faune du sol.

Les vers de terre représentent la plus grande biomasse animale terrestre et sont souvent nommés « ingénieurs de l'écosystème », de part leur contribution majeure à la pédogenèse et leur influence sur des paramètres clés du sol, comme le cycle des nutriments, la porosité du sol, et l'activité microbienne (Jones et al. 1997; Binet et al. 1998; Monard et al. 2008; Bottinelli et al. 2010). La biodiversité des vers de terre est toutefois réduite dans les champs en agriculture intensive (Smith et al. 2008), l'utilisation de pesticides étant identifiée comme un des facteurs responsables de ce déclin, d'après des études en laboratoire (Springett & Gray 1992; Yasmin & D'Souza 2010). Malgré les effets toxiques de certains pesticides, certaines

espèces persistent en agriculture intensive, en particulier les espèces endogées comme *Aporrectodea* et *Allolobophora sp* (Jordan et al. 2004; Smith et al. 2008; Pelosi et al. 2013). Ceci suggère, et c'est l'hypothèse forte de ce travail de thèse, que les impacts à long terme des pesticides sur des populations naturelles comme les vers de terre peuvent induire des phénomènes d'adaptation ou d'acclimatation chez ceux-ci. Des processus d'adaptation ont pu être mis en évidence chez des vers de terre *Dendrobaena octaedra* exposés aux métaux, facilités par une surexpression de métallothionéines (Fisker et al. 2013). Ces processus d'adaptation peuvent comporter des changements physiologiques dont une augmentation de l'activité de détoxification, des modifications d'allocation des ressources énergétiques, et des changements de comportement (réactions d'évitement), mais ils sont encore mal compris.

Objectifs de la thèse et du travail de recherche

Au cours du processus d'homologation de nouveaux pesticides, en théorie les composés les plus toxiques pour les vers de terre sont éliminés d'après les tests standardisés disponibles avec les vers de terre *Eisenia fetida* basés sur la mortalité et la reproduction. Cependant il est probable que ces tests ne prennent pas en compte des effets plus subtils des molécules appliquées :

i.) Sur les populations d'espèces plus sensibles que *E. fetida*

ii.) A long terme (plusieurs décennies)

Malgré cette contamination, dans les sols à forte activité agricole et à apports réguliers de pesticides, des populations de vers de terre de quelques espèces (3-4) persistent. Ces faits invitent à s'interroger sur les stratégies qu'ont développées ces populations pour se maintenir dans les sols contaminés par les pesticides et leurs résidus, et dans quelle mesure des processus d'acclimatation et d'adaptation sont impliqués. De plus, on peut se demander quels coûts (compromis énergétiques et reproductifs) ces stratégies peuvent avoir pour les populations (allocation d'energie et traits d'histoire de vie) mais aussi pour l'écosystème sol (efficacité de bioturbation, dynamique des cycles de carbone et d'azote, et dégradation des pesticides).

L'objectif de la thèse est donc d'évaluer ce potentiel d'adaptation chez des populations de vers de terre en comparant des populations provenant d'historiques agricoles contrastés en

terme d’applications de pesticides. En particulier, la comparaison de populations « références » (issues de zones non-contaminées, i.e. populations « naîves ») versus des populations pré-exposées (issues de champs en agriculture conventionnellle). Ce travail étudie les stratégies d'adaptation à plusieurs niveaux biologiques, du niveau cellulaire et physiologique fin (biochimique, enzymatique, métabolique) à l'individu (changements de poids) et de la population (paramètres de la reproduction), ainsi que les conséquences possibles pour l'écosystème (activité de creusement des vers de terre et conséquences pour le devenir des pesticides).

La première étape est d'évaluer dans quelle mesure l’historique agricole peut être relié à la contamination par les pesticides et les résidus de molécules dans le sol. Une analyse des résidus de pesticides est réalisée et les profils de contamination comparés entre champs afin de sélectionner les domaines les plus appropriées pour l'étude.

Une enzyme de détoxification avec une large spécificité de substrat (GST, à la fois sous forme cytosolique et microsomal) et une enzyme anti-oxydante majeure (CAT) sont utilisées et comparées au sein des populations pour évaluer si ces mécanismes de «protection» se sont amélioré dans les populations pré-exposés à pesticides par rapport à la population de référence. L’activité « basale » (c’est à dire expression constitutive des enzymes sans exposition récente aux pesticides) de ces deux paramètres est mesurée dans les vers de terre sur le terrain ainsi qu’après exposition à des concentrations susceptibles d’être rencontrées dans l'environnement (selon les taux d'application sur le terrain) en laboratoire

Les compromis énergétiques de ces mécanismes de protection seront évaluées par le statut des principaux réservoirs énergétiques, qui sont le glycogène (principal composé de stockage glucidique chez les animaux), les lipides et les protéines. Les réponses métaboliques globales des organismes seront évaluées par la mesure de la respiration, et par l'utilisation de la métabolomique. La méthodologie appliquée permet aux différentes mesures (respiration, ressources énergétiques, métabolomique) d’être réalisées sur le même animal avec plusieurs aliquots de tissu lyophilisé et broyé.

Les conséquences possibles aux niveaux supérieurs d'organisation biologique (population) sont étudiées à travers les traits d'histoire de vie. Plusieurs traits morphologiques et

biométriques sont comparés chez les adultes des différents historiques agricoles ainsi que dans leur progéniture (cocons et juvéniles).

Les conséquences de l'adaptation des vers de terre pour l'écosystème du sol sont abordées en termes de bioturbation (activité de creusement) et de dégradation des pesticides. L'activité de creusement d'une population de vers de terre pré-exposée par rapport à une population naïve est quantifiée par des expériences en laboratoire afin de déterminer le lien entre réponses adaptatives (métabolome, ressources énergétiques, respiration) et bioturbation. La dégradation des pesticides est aussi comparée entre les deux populations et dans le sol sans ver de terre (dissipation naturelle) afin de déterminer si la population pré-exposée (possiblement adaptée) peut influencer davantage la dégradation du pesticide en tant que service écosystémique.

Article 1. Tolérance des vers de terre à la contamination résiduelle par les pesticides dans les paysages agricoles : évaluation expérimentale et in situ des capacités de détoxification

L'objectif du premier chapitre est d'évaluer la capacité des vers de terre à faire face et s'adapter à une multi-contamination chronique (> 10 ans) des sols, enjeu majeur dans la prédiction de la résilience et de la durabilité de l'écosystème sol.

Les réponses de vers de terre exposés de manière chronique à la contamination par les pesticides (issus de champs en agriculture conventionnelle depuis 20 ans) sont comparées à celles de vers de terre jamais exposés (issus de champs en agriculture biologique). Dans une étude *in situ* l'hypothèse suivante est testée : la contamination résiduelle à long terme (20 ans) des sols cultivés se manifeste par des différences physiologiques chez les vers de terre. Dans la seconde partie, une exposition en laboratoire de populations pré-exposées (champ conventionnel) et naïves (champ biologique) à des concentrations en pesticides écologiquement réalistes est réalisée. Cette seconde expérience teste l'hypothèse que la pré-exposition des vers de terre à long-terme dans un champ cultivé modifie leur réponse physiologique lors d'une exposition aigüe. Les réponses physiologiques suivantes sont évaluées : capacité antioxidante (catalase), detoxification (Glutathione S Transferase), et coûts énergétiques associés (lipides, glucides, protéines).

Résultats principaux :

1. Résidus de pesticides dans les sols

9 pesticides sont détectés dans les sols des trois champs, dont 5 herbicides, 3 fongicides et un métabolite du glyphosate, l'AMPA. L'herbicide atrazine est présent dans les trois champs conventionnels plus le champ biologique, mais pas dans la prairie. C'est donc la molécule la plus fréquemment détectée, bien qu'elle n'ait pas été appliquée sur aucun des champs au cours de l'historique disponible (2000-2010). Les deux molécules utilisées actuellement et les plus fréquemment détectées sont l'alachlore et l'époxiconazole, et elles réprésentent en moyenne entre 3 et 7% de leur concentration « prédite » après une application en se basant sur la dose recommandée (Dittbrenner et al. 2010). A noter que la quantité résiduelle totale mesurée dans le sol des champs conventionnels suit le gradient pré-établi grâce à l'historique d'applications (« Forts » > « Moyens » > « Faibles »).

Table 2
Soil multiresidual pesticide contamination of the five fields studied 2011, Brittany, France - mean and standard deviations (out of three replicates) of pesticide concentrations in ng g^{-1} dry soil and number of detections (out of the 25 sampling points). In grey, pesticides that had not been applied since the start of the available cultivation history (2001). Pesticide type: h = herbicide, f = fungicide, m = metabolite, LOD = limit of detection, LOQ = limit of quantification, nd = below detection limit, <LOQ = detected but below quantification limit, total amount measured 2011 is the sum of mean concentrations, total number measured 2011 is the sum of detected molecules.

Active molecule	Type	LOD–LOQ	High input	Medium input	Low input	Organic field	Pasture	Number of detections
Atrazine	h	0.4–1.25	<LOQ	1.6 ± 0.2	2.4 ± 0.5	<LOQ	nd	17
Alachlore	h	0.8–2.5	2.9 ± 0.6	4.2 ± 0.4	8.8 ± 3.1	nd	nd	15
Epoxiconazole	f	0.8–2.5	11.1 ± 2.7	4.4 ± 1.1	4.2 ± 0.5	nd	nd	15
Cyprodinyl	f	0.8–2.5	< LOQ	<LOQ	nd	nd	nd	10
AMPA	m	16.5–50	117.5	65.9 ± 8.7	nd	nd	nd	6
Chlortoluron	h	1.7–5	nd	6.4 ± 1.8	nd	nd	nd	5
Simazine	h	0.4–1.25	nd	<LOQ	nd	nd	nd	5
Tebuconazole	f	1.7–5	nd	<LOQ	<LOQ	nd	nd	5
Azoxystrobine	h	4.2–12.5	<LOQ	nd	nd	nd	<LOQ	2
Total number measured 2011			6	8	4	1	1	
Total amount measured 2011			131.5	82.5	15.4	<LOQ	<LOQ	
Total number of molecules applied 2000–2010			17	16	19	0	0	
Total amount applied 2000–2010 (Σ kg(active ingredients)/ha)			18.2	7.5	3.2	0	0	

Table 2 : multi-contamination des sols des cinq champs étudiés en 2011. Moyennes et écarts-types (sur trois réplicats) des concentrations en pesticides

2. Activités enzymatiques *in situ* (vers de terre prélevés sur le terrain)

Globalement, l'activité des enzymes sGST et catalase chez A. caliginosa, ainsi que mGST chez A. chrlorotica augmentent avec l'apport de pesticides dans les champs conventionnels, et sont les plus faibles dans le champ bio et la prairie (Figure 1).

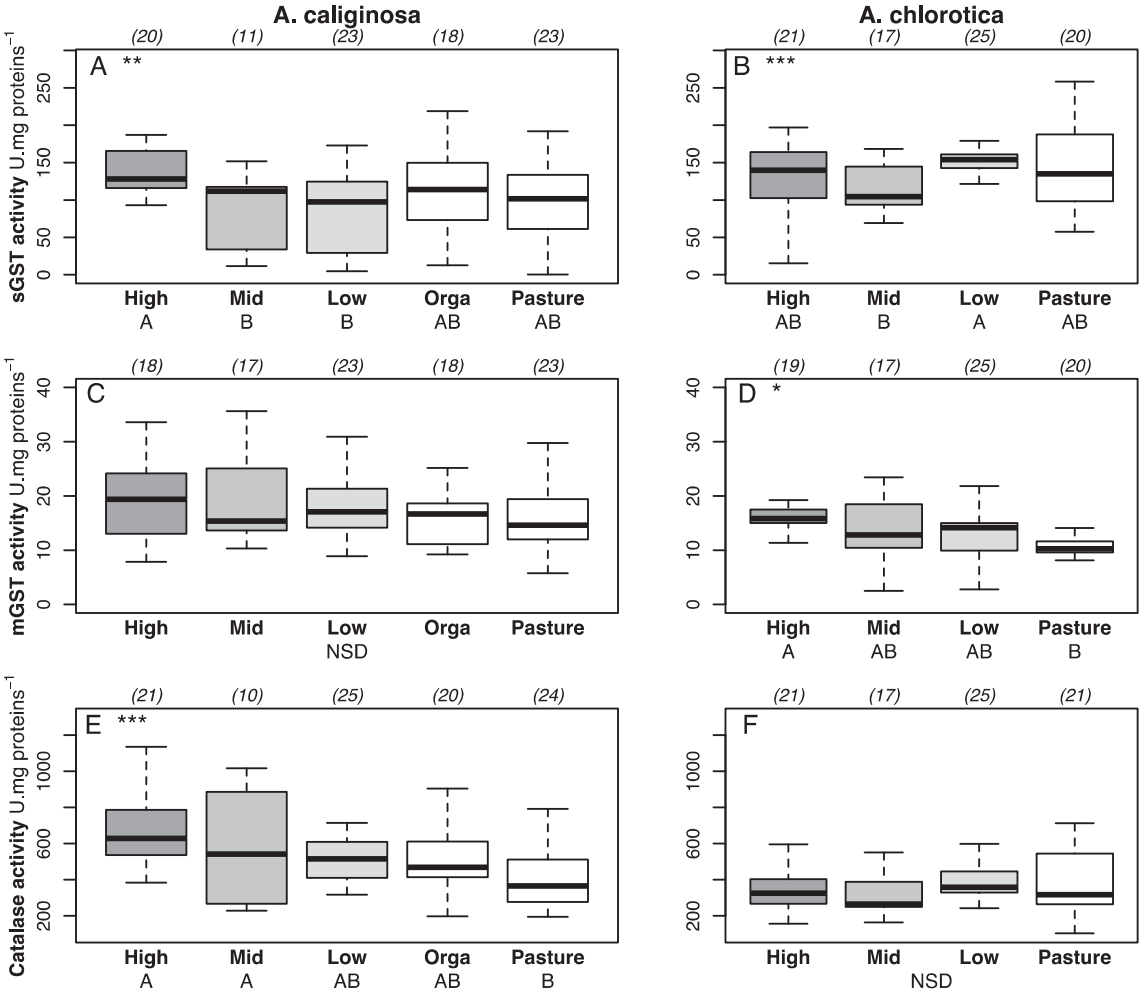

Figure 1 : activités enzymatiques dans les vers de terre selon l'historique de culture : conventionnel à intrants « Forts » (High), Moyens (Mid) et bas (Low) », organique cultivé (Orga) et organique prairie (Pasture). Activités de la Glutathion S Transferase soluble (sGST) et microsomale (mGST), et de la catalase (CAT) dans les vers de terre *A. caliginosa* (graphes de gauche) et *A. chlorotica* (graphes de droite). Un impact significatif de la contamination du sol (Anova à un facteur « champ ») est indiqué par des astérisques en haut à gauche des graphes. Au dessus de chaque graphe, les nombres d'individus (réplicats) pour chaque modalité sont indiqués entre parenthèses.

3. Réponses enzymatiques après exposition à deux pesticides et à leur mélange.

Au début de l'expérience, les groupes témoins des deux populations de vers *A. caliginosa* (issus du conventionnel « hauts intrants » et du champ biologique) ont des activités enzymatiques similaires. Après exposition, l'activité de la Glutathion S Transférase soluble (sGST) est très différente entre les deux populations. L'activité de la sGST augmente significativement avec le traitement epoxiconazole après 7 et 28 jours d'exposition chez la population pré-exposée (conventionnel « hauts intrants »), mais pas chez la population naïve (Figure 2). Le glyphosate ne provoque pas de changements entre témoins et exposés. Le mélange glyphosate/epoxiconazole augmente toutefois l'activité de la sGST après 28 jours d'exposition. L'activité de la Glutathione S Transferase microsomale n'est quasiment pas modifiée par l'époxiconazole, mais elle diminue significativement avec l'herbicide et le mélange des deux pesticides.

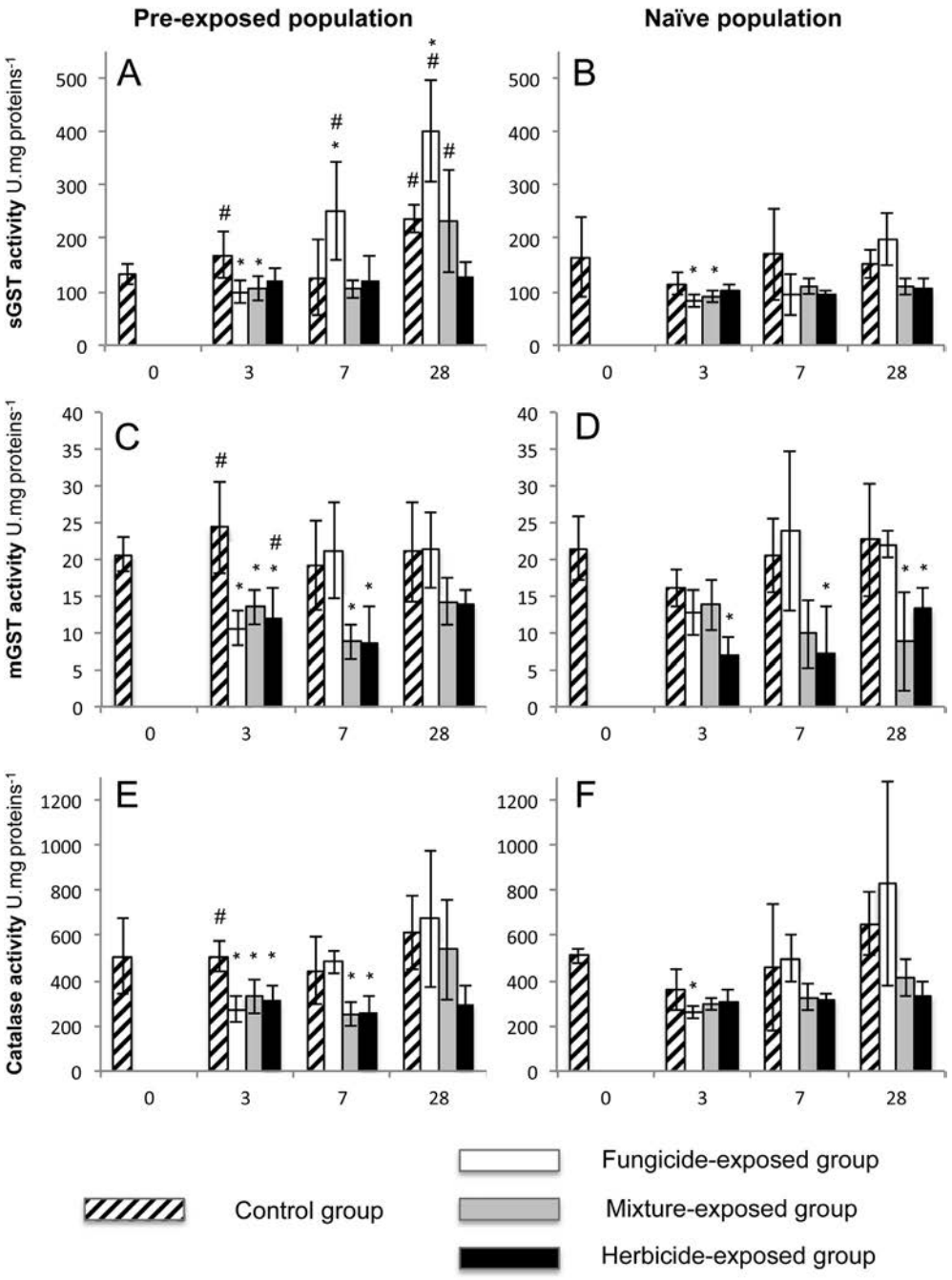

Figure 2 : capacités de détoxification chez des vers de terre pré-exposés A. caliginosa après exposition en laboratoire. Les histogrammes représentent les activités, moyennes de 6 réplicats, des enzymes Glutathione S transferase (soluble et microsomale) et catalase dans les vers de terre A. caliginosa après 3, 7, et 28 jours d'exposition au fongicide OPUS (0.1 µg molécule active / g sol sec), à l'herbicide Round Up Flash (2.5 µg molécule active / g sol sec) et leur mélange (somme des deux précédentes concentrations). * représente une différence significative entre groupe exposé à un pesticide et témoin (sans pesticide).

Article 2: adaptation de vers de terre aux pesticides en paysage agricole, mécanismes physiologiques et conséquences écosystémiques

L'objectif de ce deuxième chapitre est de tester l'hypothèse selon laquelle des vers de terre issus d'un sol agricole conventionnel (avec utilisation de pesticides) ont acquis une tolérance vis à vis du fongicide époxiconazole, fréquemment appliqué sur le champ.

Les réponses en laboratoire de vers de terre issus d'une population pré-exposée (champ conventionnel) sont comparées à celles d'une population naïve (champ en agriculture biologique) après exposition à une dose "environnementalement pertinente" du fongicide (correspondant au taux d'application par hectare). Ce travail s'attache à déterminer si une adaptation est quantifiable en comparant stockage énergétique et métabolisme (respiration et métabolomique) dans les deux populations.

Design expérimental:

Table 1
Experimental design of the laboratory exposure. Out of each group of 11 microcosms containing one earthworm, 5 randomly picked worms were used for respirometry assessment, energy resources, and metabolite measurements.

		Worms at start	Sampling days and number of worms sampled			Treatment
			0	7	28	
Microcosms containing one earthworm	Pre-exposed population	33	11	11	11	CTRL
	(conventional field)	22	0	11	11	EPOXI
	"Naïve" population	33	11	11	11	CTRL
	(organic field)	22	0	11	11	EPOXI
Microcosms without earthworm	Control microcosms for cast production	0	8	8	8	CTRL
	Control microcosms for pesticide dissipation	0	3	3	3	EPOXI
	Control microcosms for humidity check	0	3	3	3	CTRL

Table 1: Design expérimental de l'expérience de laboratoire. Dans chaque groupe initial de 11 vers de terre (11 microcosmes), l'activité de bioturbation est mesurée dans 8 sur 11, la concentration en pesticides dans les trois micrososmes restants, et 5 vers sont pris au hasard pour les analyses de respirométrie, ressources énergétiques, et métabolomique

Principaux résultats:

- Respiration et dissipation énergétique

La figure 2 (ci-dessus) montre une activation de la production de CO2 dans les deux populations suivant l'exposition à l'époxiconazole après 7 et 28 jours comparé aux groupes témoins non-exposés. Une différence significative entre les deux populations apparaît après 28 jours, la respiration est plus importante chez la population pré-exposée.

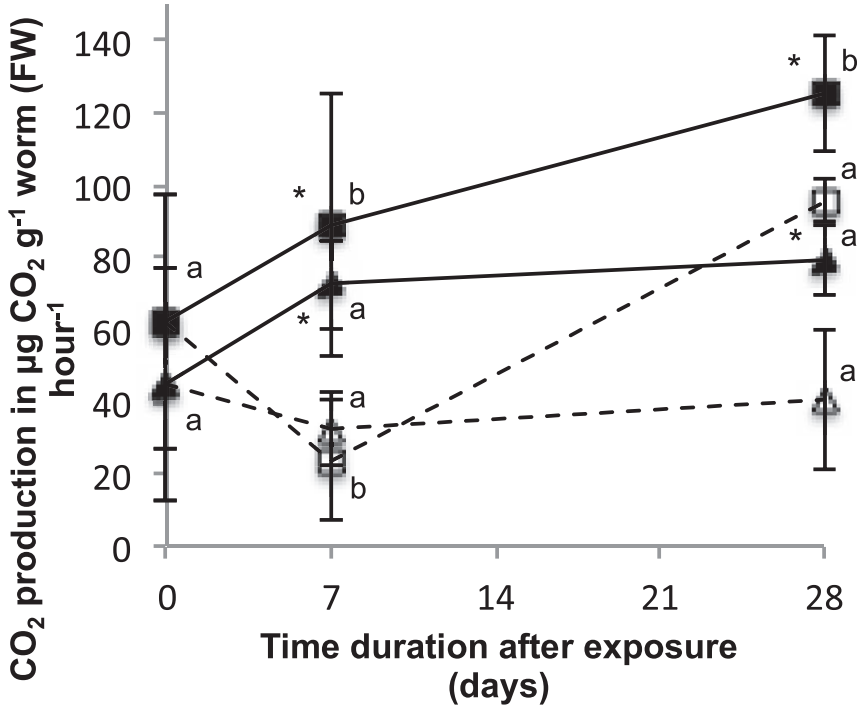

Figure 2: taux métabolique (µg CO2 . g^{-1} . ver^{-1} (poids frais) dans les populations de vers de terre pré-exposées (carrés) et naïves (triangles) après exposition à l'époxiconazole. Les symboles noirs sont les groupes exposés au fongicide et les blancs sont les groupes témoins (sans pesticides). Les résultats sont les moyennes sur 5 individus ± écarts types (barres d'erreurs). Les différences significatives entre groupes exposés et témoins sont indiqués par * et par # entre populations. Des lettres a et b différentes marquent les différences entre dates d'échantillonnages au sein du même groupe

- Production de turricules (bioturbation)

La figure 3 (ci-dessous) montre qu'après 7 jours, l'application d'époxiconazole induit une augmentation significative de la production de turricules chez la population pré-exposée uniquement.

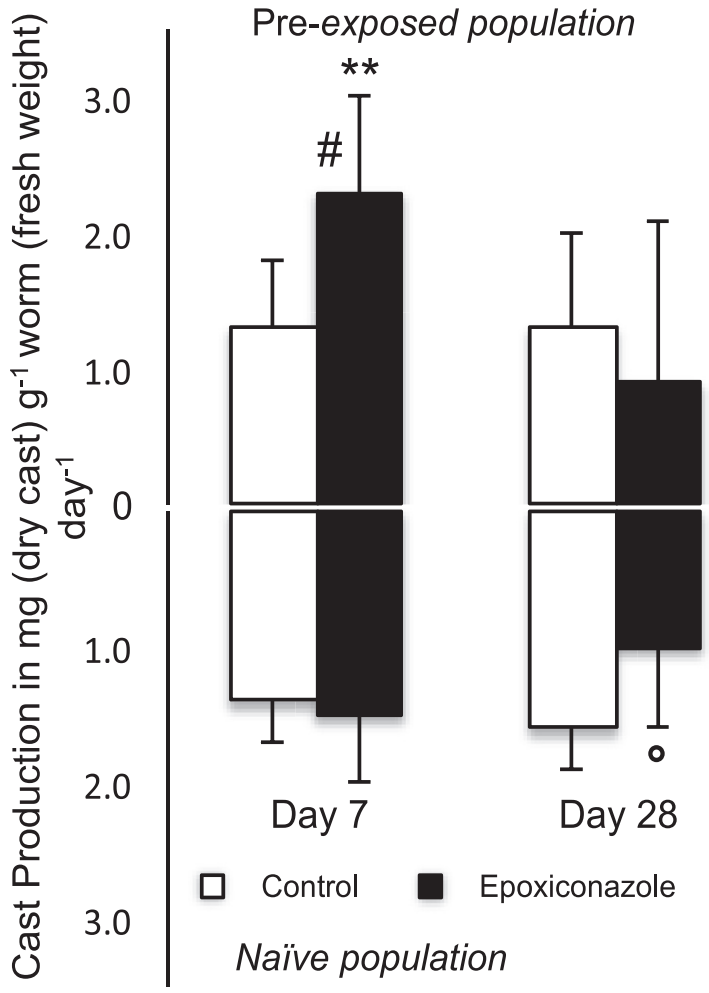

Figure 3: production moyenne de turricules (en g de turricule sec par g de masse fraîche de ver de terre) chez deux populations de vers de terre naïve (en dessous) et pré-exposée (au dessus) après exposition en laboratoire à l'époxiconazole après 7 et 28 jours (n=8). Les barres d'erreurs sont les écarts-types. * indique une différente significative ($p<0.05$) entre groupe exposé au fongicide et témoin (sans pesticide) et # entre les deux populations.

- Ressources énérgétiques

La figure 4 ci-dessous montre que le taux de glycogène baisse d'environ 20 mg dans les groupes exposés aux fongicides par rapport aux témoins après 7 jours chez les vers de terre naïfs et après 28 jours chez les vers de terre pré-exposés. Les niveaux de lipides varient peu au cours des 28 jours (pas de différences significatives). Les concentrations en protéines augementent significativement dans les deux populations par rapport aux témons après 28 jours, mais les vers de terre pré-exposés ont un taux de protéines final presque deux fois plus élevé que les les animaux naïfs.

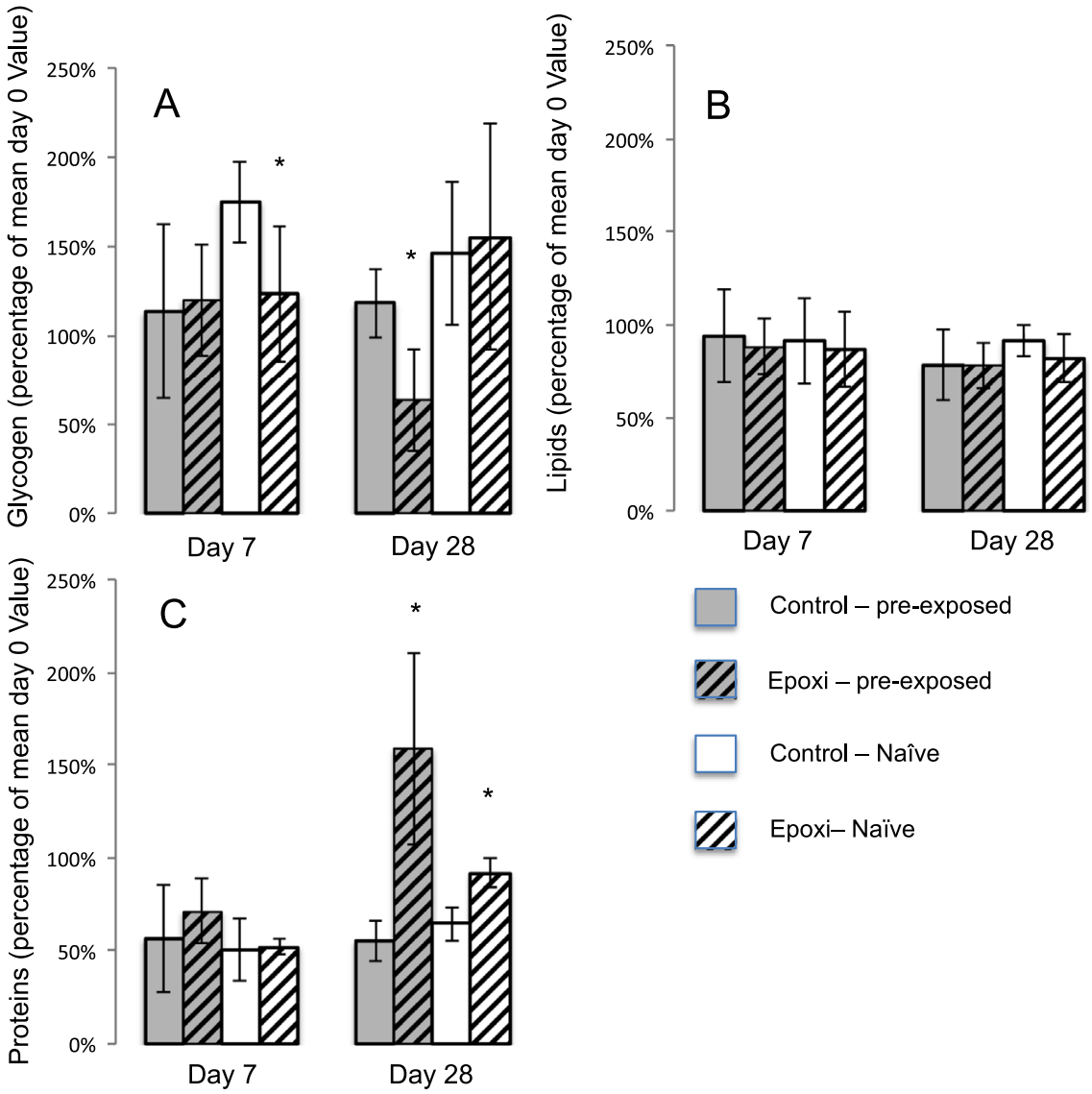

Figure 3: taux de glycogène (A), lipides (B) et protéines (C) chez des populations de vers de terre *Aporrectodea caliginosa* pré-exposées et naïves après exposition au fongicide epoxiconazole. Les résultats sont exprimés en pourcentage du taux intitial (début de l'expérience à 0 jours), et sont des moyennes sur 5 individus ± écarts-types (barres d'erreur). Les différences significatives sont indiquées par * entre groupes exposés au fongicide et témoins.

- Métabolomique

28 métabolites sont détectés dans les tissus des animaux. 22 métabolites pertinents pour le métabolisme énergétique sont gardés sur les 28 pour interprétation des résultats. La figure 4 montre la représentation graphique d'une analyse en composantes principales du jeu de données métabolomique. Les scores des différents individus sur les composantes deux et trois (population « pré-exposée » = agriculture conventionnelle) et 1 et 2 (population « naïve » =

agriculture biologique) sont représentés en A et B en fonction du temps d'exposition. Chez la population conventionnelle, les vers de terre exposés pendant 28 jours forment un groupe séparé des vers témoins selon les axes 2 et 3. Ce fait n'est pas observé chez la population biologique ou on constate un effet du temps d'exposition mais pas du fongicide.

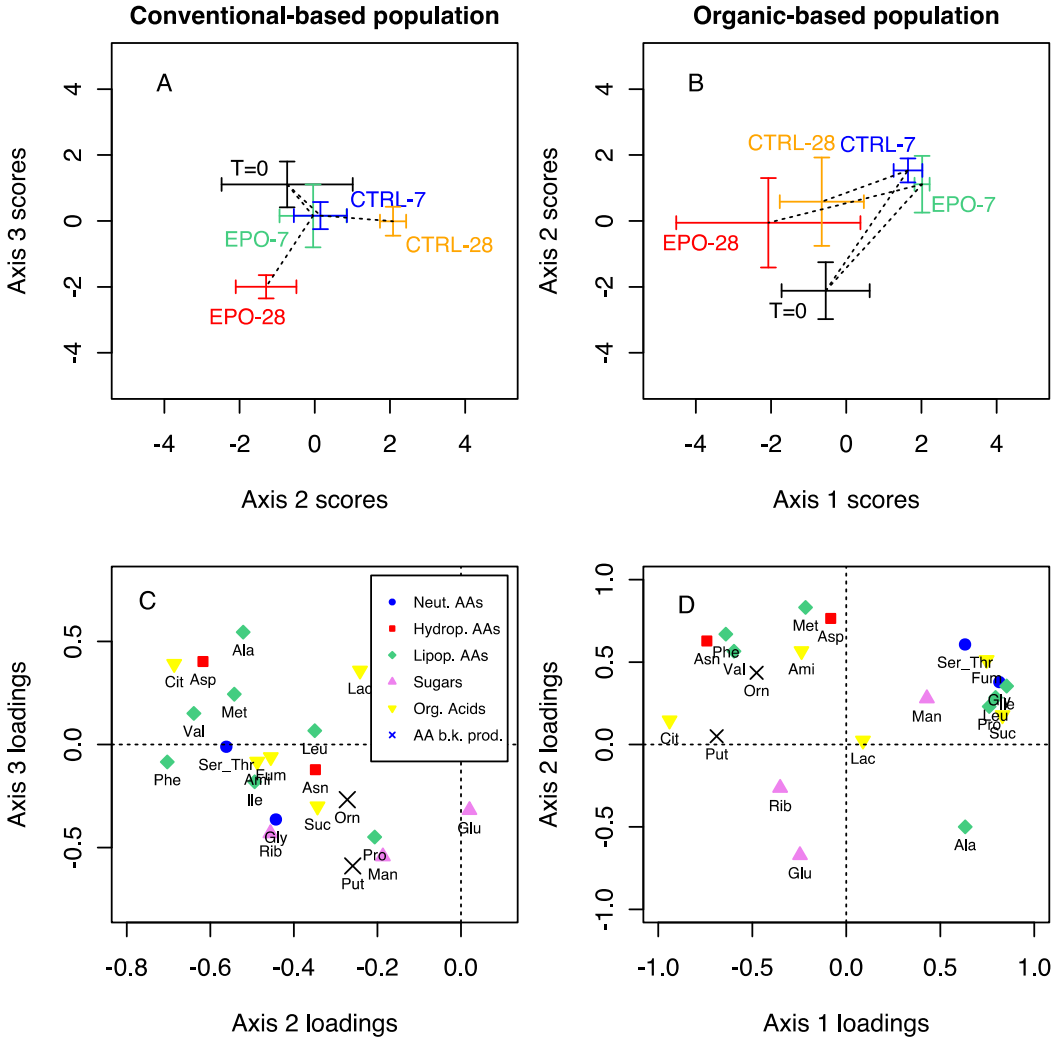

Figure 4: analyse en composantes principales du jeu de données métabolomique (22 variables) montrant les relations et corrélations entre les profils métaboliques et l'exposition à l'époxiconaezole. A et B: scores des individus sur les composantes principales (2 et 3 pour la population conventionnelle et 1 et 2 pour la population biologique). Les données sont représentées comme moyennes (croix) des scores sur les deux composantes principales pour les groupes exposés et contrôles ± erreur standard de la moyenne. Les groupes exposés et contrôles sont joints par une ligne pointillée par ordre de temps.

C et D: saturations des variables avec les composantes principales (corrélations entre variables et composantes principales).Les métabolites sont identifiés par leur abbréviation et colorés par groupes fonctionnels (voir légende).

Article 3 : traits d'histoire de vie et réponse à la contamination du sol chez des populations de vers de terre issus de gestions agricoles contrastées

L'objectif de ce chapitre est de tester l'hypothèse selon laquelle des vers de terre issus d'un sol cultivé depuis longtemps en agriculture conventionnel ont pu développer des réponses adaptatives aux pesticides en terme de traits de vie.

Les traits de vie sont comparés dans deux populations indépendantes de ver de terre *Aporrectodea caliginosa* issues d'un champ en agriculture conventionnelle (avec pesticides) et d'un champ en agriculture biologique (sans pesticides), depuis au moins 20 ans. Les différences entre populations sont étudiées en terme de poids des adultes, juvéniles et cocons, epaisseur de la paroi du cocon, temps et taux d'éclosion des cocons, et taux de croissance des juvéniles après exposition aux polluants « modèles » glyphosate (herbicide) et époxiconazole (fongicide).

Résultats principaux (traits de vie) :

La table 2 montre les traits de vie des deux populations (adultes issus des champs et génération suivante).

		Population and champ d'origine	
		pré-exposée conventionel	naïve biologique
Poids	Adultes	417 ± 171 mg	**662 ± 193** mg
	Cocons	12.50 ± 2.30 mg	**13.72 ± 6.30** mg
	Nombre de juvéniles	12.24 ± 3.32 mg	11.04 ± 3.39 mg
	Paroi du cocon	0.54 ± 0.38 mg	**2.39 ± 0.31** mg
Eclosion	Temps d'éclosion	30.71 ± 3.96 jours	**28.34 ± 5.10** jours
	Taux d'éclosion	64 %	**81** %
	Survie des juvéniles	48 %	48 %
Paroi du cocon	Epaisseur	24.82 ± 4.02 µm	24.61 ± 4.98 µm

Table 2: traits de vie de populations de vers de terre *Aporrectodea caliginosa* (n=150) issus d'un champ conventionnel "pré-exposée" et d'un champ biologique "naïve". Poids moyens des adultes et des cocons produits, nombre de juvéniles moyens, différence moyenne "cocon produit moins juvénile éclos ", temps et taux d'éclosion moyen des cocons, survie des juvéniles, et épaisseur moyenne des cocons par population. Les différences significatives entre populations sont indiquées en gras.

Les poids moyens des vers adultes et de leurs cocons sont significativement plus faibles dans le champ conventionnel que dans le champ biologique. Le temps d'éclosion est aussi significativement plus long et le taux d'éclosion plus faible pour les cocons issus de la population conventionnelle.

Discussion et conclusions

Il y a actuellement un manque d'informations disponibles sur la contamination résiduelle des sols par les pesticides. Les pesticides, bien qu'ils soient appliqués sur les cultures, ne sont pas souvent mesurés dans le sol, mais plutôt dans les plantes et les eaux souterraines (Van-Camp et al. 2004). Aucune mesure détaillée des pesticides dans les sols ne sont disponibles à l'échelle de l'UE pour évaluer leur contamination résiduelle. La modélisation permet cependant d'estimer de façon plus concrête ces résidus dans les sols (European Commission 2004). Un modèle simple de contamination par les herbicides dans les sols des pays européens a été réalisée en 2004 par l'Unité de gestion des ressources foncières de la Commission européenne (2004). Le modèle prédit que, avec les pratiques agricoles actuelles , dans plusieurs pays de l'UE dont la France, les quantités d'herbicides dans les sols cultivés en céréales, maïs et betterave à sucre sont susceptibles d'augmenter. En se basant sur l'histoire de la culture des trois champs conventionnels dont fait l'objet ce travail de thèse, plus de 50 molécules actives de pesticides différentes ont été appliquées à différents taux d'application et fréquences entre 2000 et 2010. Cependant, seulement 9 d'entre eux ont été détectées ou quantifiées dans le sol en 2011, indiquant des persistences dans les sols très variables (manuscrit 1). Une grande diversité de molécules est détectée dans les sols des champs conventionnels, et seul l'herbicide atrazine est détecté dans le champ en agriculture biologique, indiquant probablement une contamination ancienne. De nombreuses molécules appliquées récemment (2005-2010) sur les champs conventionnels ne sont pas détectées indiquant une dégradation rapide. Toutefois cette étude permet de souligner la persistance des molécules atrazine, époxiconazole, et alachlore. En effet, ils ont des demi-vies assez longues dans le sol : 146 jours pour l'atrazine (Pesticide Action Network), 400 jours (in situ) pour l'époxiconazole (Bromilow et al. 1999), et 20 jours pour l'alachlore en sol oxygéné (Pesticide Action Network). Une capacité de détoxification augmentée et un plus grand potentiel anti-oxidant sont observés le long du gradient de contamination du sol et en laboratoire après exposition des vers de terre des champs conventionnel (population « pré-exposée ») et biologique (« naïve) à des pesticides.

Des résidus de pesticides sont donc détectés dans les sols longtemps après leur dernière application sur les champs (jusqu'à plus de 20 ans pour l'herbicide atrazine). Une corrélation est observée entre l'historique d'exposition agricole des vers de terre aux pesticides et leur capacité de détoxification (in situ et en laboratoire). Des différences spécifiques entre espèces en terme d'activité de détoxification in situ sont aussi constatées entre *Aporrectodea caliginosa* et *Allolobophora chlorotica.* La corrélation entre activité de détoxification et anti-oxydante et la contamination des sols est démontrée uniquement chez *A. caliginosa.*

Cette étude est la première démontrant une réponse adaptative (mais non-génétique) dans le système de désintoxication de vers de terre exposés aux pesticides à long terme. Cette réponse adaptative pourrait non seulement être fondée sur la Glutathion-S-Transferase (mécanisme de détoxification de phase II), mais peut-être aussi sur d'autres phases du système biotransformation / détoxication, tels que les monooxygénases cytochrome p450-dépendantes, c'est-à-dire la désintoxication de phase I. L'augmentation marquée des niveaux de protéines solubles dans les vers de terre pré-exposés lorsqu'exposés au fongicide soutient l'hypothèse que d'autres parties du système de détoxication pourraient être impliqués. La mesure d'enzymes de détoxification supplémentaires telles que le cytochrome P4501A (CYP1A) monooxygénase (Lukkari et al. 2004) permettrait de préciser ce mécanisme adaptatif. Les bases génétiques du mécanisme restent aussi à démontrer. Par exemple, d'autres essais d'exposition au fongicide époxiconazole, et éventuellement à d'autres pesticides (pour tester si cette réponse est spécifique à un polluant ou à une famille de composés) pourraient aussi être réalisés avec des générations F1 et F2.

Une adaptation physiologique est démontrée chez les animaux pré-exposés, qui est associée à une 'augmentation de la bioturbation, et en cascade à une dissipation du pesticide dans le sol. Les conséquences au niveau de la population sont observées en termes de traits d'histoire de vie des deux populations pré-exposées et naïves. Le management en conventionnel incluant l'utilisation de pesticides semble diminuer le poids des adultes au champ, et implique potentiellement la réallocation des ressources énergétiques, des mécanismes reproductifs vers les processus métaboliques. Ceci aboutit à une diminution de la fécondité et du pourcentage d'éclosion et pourrait être un facteur participant à la diminution des populations de vers de terre dans les champs cultivés avec utilisation de produits phytosanitaires.

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PhD Thesis by

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Adaptation strategies of soil biodiversity (earthworms) to pesticides: mechanisms in play and ecosystemic cost assessment

to obtain the degree of Doctor (PhD) in Biology

as joint degree ("cotutelle") between the University of Rennes 1 and the University of Southern Denmark

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A mon grand-père, Claude Givaudan

Table of contents

Preface

This PhD thesis presents work conducted both at the University of Rennes 1 in the "ECOBIO" unit (UMR CNRS-ECOBIO 6553) and at the University of Southern Denmark, Institute of Biology. It was realized through a joint degree ("cotutelle") between both Universities. It was funded by the European University of Brittany (UEB) in the frame of the International Chair of Excellence in Agronomy and Environment granted to Claudia Wiegand and to ECOBIO laboratory, and by the Institut Français du Danemark. It is also part of the LIA "Environmental Toxicology and Stress Ecology" sustained by the Environmental and Ecology Institute of CNRS (INEE-CNRS), the University of Southern Denmark (SDU) and the University of Rennes 1.

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Manuscripts included in this Thesis

1. **Givaudan N.**, Binet F., Le Bot B., Wiegand C. Earthworm tolerance to residual pesticide contamination in agricultural landscapes : from field assessment to physiological responses. *Environmental Pollution.* Submitted (2014)

2. **Givaudan N.**, Wiegand C., Le Bot B., Renault D., Pallois F., Llopis L., Binet F. Acclimation of earthworms to chemicals in anthropogenic landscapes, physiological mechanisms and soil ecological implications. *Soil Biology and Biochemistry*. Accepted (28-01-2014)

3. **Givaudan N.**, Wiegand C., Martineau B., Llopis S., Binet F. Life traits comparison of earthworm cocoons in populations originating from different agricultural practices. *European Journal of Soil Sciences.* In preparation (2014)

Participations to conferences and workshops

1. Givaudan N., Wiegand C., Renault D., Lebot B., Martineau B., Llopis S., Binet F. Adaptation strategies of soil biodiversity (earthworms) to pesticides: physiological mechanisms and soil ecological implications (*Platform presentation*). *Conference on Pesticide Behaviour in Soils, Water and Air*. Conference held in York, 2-4 September 2013.

2. Givaudan N., Wiegand C., Le Bot B., Binet F. Earthworm tolerance to residual pesticide contamination in agricultural landscapes : from field assessment to physiological responses (*Poster). 2nd European Doctoral College on Environment and Health (EDCEH) 4-6 June 2012*, Rennes

3. Givaudan N., Binet F., Martineau B., Llopis S., Wiegand C. Energetic patterns and burrowing activity after exposure to two pesticides in reference and pesticide-exposed earthworm populations (*Poster).* Workshop held in Paimpont, France, 21-23 november 2012: *Genericity of responses and adaptation strategies to multiple stresses in organisms of aquatic and terrestrial ecosystems*

4. Givaudan N., Wiegand C., Binet F. Potentiel d'adaptation des lombricidés aux pollutions environnementales résiduelles établies en paysage agricole (*Platform presentation*). Colloque des Zones Ateliers, CNRS. Colloqium held on 4-7 october 2011, Rennes

Additional articles

1. Jaffal, A., Givaudan, N., Betoulle, S., Terreau, A., Paris-Palacios, S., Biagianti-Risbourg, S., Beall, E., Roche, H., 2011. Polychlorinated biphenyls in freshwater salmonids from the Kerguelen Islands in the Southern Ocean. *Environmental Pollution* 159, 1381–1389.

2. Sotton, B., Devaux, A., Givaudan, N., Guillard, J., Domaizon, I., Bony, S., Anneville, O., 2012. Short-term uptake of microcystin-LR by Coregonus lavaretus: GST activity and genotoxicity. *Ecotoxicology* 21, 1788–1796.

3. Sotton, B., Guillard, J., Bony, S., Devaux, A., Domaizon, I., Givaudan, N., Crespeau, F., Huet, H., Anneville, O., 2012. Impact of Toxic Cyanobacterial Blooms on Eurasian Perch (Perca fluviatilis): Experimental Study and In Situ Observations in a Peri-Alpine Lake. *PLoS ONE* 7, e52243.

Abbreviations

AMPA	2-amino-3-(5-méthyl-3-hydroxy-1,2-oxazol-4-yl) propanoic acid
ANOVA	Analysis of Variance
CAT	Catalase
CEC	Cation-Exchange Capacity
CT	Conventionnal Tillage
CYP	Cytochromes P-450
DNA	deoxyribonucleic acid
EC_{50}	Effective Concentration for 50% of the individuals
ERA	Ecological Risk Assessment
EROD	ethoxyresorufin-O-deethylase
GST	Glutathione S-Transferase
HSP	Heat-Shock Proteins
IQR	Inter-Quartile Range
ISO	International Organization for Standardization
LC	Layer Cultivation
LC-MS	Liquid Chromatography Coupled to Mass-Spectrometry.
LC_{50}	Lethal concentration for 50% of the individuals
LOD	Limit of Detection
LOQ	Limit of Quantification
LP	Two-layer ploughing
MCPA	2-methyl-4-chlorophenoxyacetic acid
mGST	Microsomal Glutathione S-Transferase
NOEC	No-Observed-Effect Concentration
NRC	National Research Council
NRRT	Neutral Red RetentionTime
OECD	Organisation for Economic Cooperation and Development
P	Ploughing
PAH	Polycyclic Aromatic Hydrocarbons
PCA	Principal Component Analysis
PCB	Polychlorobiphenyls
PPP	Plant Protection Products
ROS	Reactive Oxygen Species
RT	Reduced Tillage
SD	Standard Deviation
sGST	Soluble Glutathione S-Transferase
WBEB	Whole-Body Energy-Budget

Summary of the thesis (English)

This work investigated if long-term residual contamination of agricultural soils leads to adaptation of earthworm populations to pesticides. It also aimed at identifying the costs of adaptation from the individual to the population level, and the consequences for the ecosystem. Residual contamination by pesticides was assessed and compared in three fields under conventional management (classified after evaluation of pesticide applications as "high-", "medium-", and "low-"pesticide input), one field under organic agriculture requirements and one organic permanent pasture, all in this type of management for more than 20 years. Using a water extraction method, as indicative of the amount of bioavailable pesticides 6, 8 and 4 residues of pesticides were recovered in the "high-", "medium-", and "low-input" fields, respectively, and almost no pesticides were detected in the soil of the organic field except for low levels of residual (possibly 20 years old) atrazine. The endogeic species *Aporrectodea caliginosa* and *Allolobophora chlorotica* were found in common to the five fields, -except *A. chlorotica* which was absent from the organic field-, and were used as biological models. Adaptation strategies were investigated by comparing the populations of these earthworms between the different fields according to several endpoints in field and laboratory assessments. The endpoints measured ranged from the molecular (biotransformation and anti-oxidant enzymes), biochemical (main energy resources), and metabolic (respiration rate, metabolomics) levels, to individual (weight, length) and population-related parameters (cocoon and juvenile life traits), and to the possible consequences for the ecosystem in terms of bioturbation (earthworm burrowing behaviour) and pesticide disappearance as an crucial ecosystem service. Enhanced detoxification and anti-oxidant potential was demonstrated along the gradient of contamination in the fields, and in particular comparing the response to an experimental pesticide exposure between the pre-exposed, thus possibly adapted earthworms from the "high-input" field-, and the naïve population from the organic field. Distinct energetic demands and metabolic rearrangements were observed between the populations, more pronounced in the pre-exposed earthworms. Physiological adaptation was demonstrated in pre-exposed animals, and this was associated with an increase in burrowing behaviour and pesticide disappearance in the soil. Population-level consequences were assessed in life traits of the two populations. The conventional farming including the use of pesticides decreased the weight of adult worms in the field and resulted in reallocation of energy resources, possibly from reproductive to metabolic function. This led to lower fecundity and hatching success and could partly explain lower earthworm densities in pesticide-impacted soils.

Keywords : *Aporrectodea caliginosa*, pesticides, biotransformation, oxidative stress, adaptation, Land-use, Epoxiconazole, Energy storage, Metabolomic profile, Soil bioturbation, Life history traits, cocoons, growth, pesticides, soil organic contamination

Summary of the thesis (Danish)

I dette arbejde blev der forsket i om langsigtet residualforurening af landbrugsjord fører til pesticidadaptation hos regnorme. Forskningen sigtede også efter at identificere adaptations-omkostningerne fra individ- til populationsniveau, og konsekvenserne for økosystemet. Pesticiders residualforurening blev vurderet og sammenlignet i; tre konventionelt styrede marker (klassificeret efter evaluering af pesticid-anvendelser som ”højt-”, ”medium-”, og ”lav-input”), en mark styret ud fra økologiske betingelser, og et økologisk permanent græsningsareal, alle havde været styret på denne måde i mere end 20 år. Ved hjælp af en vand-ekstraktionsmetode blev 6,8 og 4 pesticidresiduums udvundet i henholdsvis ”høj-”, ”medium-”, og ”lav-input” markerne, og næsten ingen pesticider blev detekteret i jorden fra den økologiske mark bortset fra lave niveauer af resterende (højst sandsynligt 20 år gammelt) atrazin. Regnorms-populationerne *Aporrectodea caliginosa* og *Allolobophora chlorotica* blev fundet i alle fem marker, bortset fra *A. chlorotica* som ikke var til stede i den økologiske mark. Adaptationsstrategier blev undersøgt ved at sammenligne populationerne af disse regnorme de forskellige marker imellem ifølge flere parametre i felt- og laboratoriebedømmelserne. De målte parametre rangerede fra molekylære- (biotransformation og antioxiderende enzymer), biokemiske- (primære energiressourcer), og metaboliske- (respirationsrate, metabolomics) niveauer, til individuelle- (vægt, længde) og populations-relaterede parametre (puppe og juvenile livskarakterer), og til de mulige konsekvenser for økosystemet med hensyn til bioturbation (regnormes grave-adfærd) og nedgang i koncentrationen af ekstraherbare pesticider. Forbedret afgiftnings- og antioxiderende potentiale blev demonstreret langs gradienten af pesticid forurening i felten. Forbedret afgiftning i den pre-eksponerede population var særdeles tydeliggjort ved eksponeringen til pesticider i laboratoriet sammenliget med den økologiske population. Der blev observeret distinkte energibehov og metabolisk omgruppering populationerne imellem, dette var mere udtalt hos de pre-eksponerede regnorme. Der blev detekteret fysiologiske adaptationer hos de pre-eksponerede dyr, og dette hang sammen med en kompensatorisk øgning i grave-adfærd og nedgang i koncentrationen af ekstraherbare pesticider i jorden. Konsekvenser, på populationsniveau, blev bedømt ud fra de to populationers livskarakterer. Brug af pesticider resulterede i vægtnedgang hos de voksne orme i felten og omfordeling af energiressourcer, formodentlig fra reproduktiv til metabolisk funktion. Dette førte til lavere frugtbarhed og udklæknings-succes og kunne til dels forklare de lavere regnorme-densiteter i den pesticid-påvirkede jord.

Keywords : *Aporrectodea caliginosa*, pesticider, biotransformation, oxidativ stres, adaptation, Epoxiconazole, Energi oplagring, Metabolomisk profil, jord bioturbation, livshistorie, kokoner, vækst, organisk jord forurening

Summary of the thesis (French)

Ce travail de thèse a cherché à déterminer si la contamination résiduelle à long terme des sols agricoles par les pesticides induit le développement de mécanismes d'adaptation aux pesticides chez les vers de terre. Il a aussi visé à identifier les coûts potentiels de l'adaptation de l'échelle de l'individu à celle de la population, et les conséquences pour l'écosystème sol. Une contamination résiduelle du sol par les pesticides est mesurée et comparée dans trois champs cultivés en agriculture conventionnelle (classés en fonction de l'historique cultural comme « haut », « moyen » et « bas » niveaux d'intrants), un champ cultivé en agriculture biologique et une prairie permanente biologique, tous dans ce type de management agricole depuis plus de 20 ans. En utilisant une méthode d'extraction des pesticides en milieu aqueux (représentant la fraction « biodisponible » des pesticides), 6, 8 et 4 résidus de pesticides sont détectés dans les champs à « haut », « moyen », et « bas » niveaux d'applications, respectivement, et un seul pesticide dans le sol du champ biologique (un résidu d'atrazine potentiellement vieux de plus de 20 ans). Les deux espèces endogées *Allolobophora chlorotica* et *Aporrectodea caliginosa* , communnes dans les sols des 5 champs,- mis à part *A. chlorotica* qui est absente du champ cultivé en agriculture biologique-, ont servi de modèles biologiques d'étude. Les stratégies d'adaptation aux pesticides sont étudiées en comparant les réponses de ces populations de vers de terre sur le terrain et après des expositions aux pesticides en laboratoire. Les réponses mesurées s'étendent de l'échelle moléculaire (enzymes de biotransformation et du stress oxidatif), biochimique (ressources énergétiques), métabolique (taux de respiration, métabolomique) à l'échelle de l'individu (biomasse, longueur) et de la population (traits de vie des cocons et des juvéniles), et aux possibles conséquences pour l'écosystème sol en termes de bioturbation (creusement et ingestion de sol) et de dissipation des pesticides comme service ecosystémique. Une capacité de détoxification augmentée et un plus grand potentiel anti-oxidant sont observés le long du gradient de contamination du sol et en laboratoire après exposition des vers de terre des champs conventionnel (population « pré-exposée ») et biologique (« naïve) à des pesticides. Des demandes énergétiques et des réarrangements métaboliques différents sont observés dans les deux populations, et sont plus prononcés chez la population pré-exposée. Une adaptation physiologique est démontrée chez les animaux pré-exposés, qui est associée à une 'augmentation de la bioturbation, et en cascade à une dissipation du pesticide dans le sol. Les conséquences au niveau de la population sont étudiées en termes de traits d'histoire de vie des deux populations pré-exposées et naïves. Le management en conventionnel incluant l'utilisation de pesticides semble diminuer le poids des adultes au champ, et implique potentiellement la réallocation des ressources énergétiques, des mécanismes reproductifs vers les processus métaboliques. Ceci aboutit à une diminution de la fécondité et du pourcentage d'éclosion et pourrait être un facteur participant à la diminution des populations de vers de terre dans les champs cultivés avec utilisation de produits phytosanitaires.

Mots-clés : *Aporrectodea caliginosa*, pesticides, biotransformation, stress oxidatif, adaptation, Epoxiconazole, ressources énergétiques, profil métabolique, bioturbation du sol, traits d'histoire de vie, cocons, croissance, contamination des sols

"It may be doubted whether there are many other animals which have played so important a part in the history of the world, as have these lowly organised creatures."

Darwin, 1881

General introduction

1 The ecological role of soil biodiversity

1.1 The functional domains of the soil

The soil is an heterogeneous medium led by a hierarchy of both biotic and abiotic determinants acting at different scales of time and space. In order of decreasing time and space scale the major determinants are climate, soil properties, organic matter quality and organisms (2007). Soil processes are mainly sustained by the biota and its biological activities. The different components of the soil biota produce structures which are dependent on the organisms and the communities that produced them (Lavelle 2002). The assumption that structures created by a species, a group of species or abiotic factors can be identified and separated from the soil volume led to the definition of soil functional domains. Functional domains are thus parts of the soil that are influenced by a major biotic or abiotic factor, and that could be physically separated from the rest of the soil matrix (Lavelle 2002). They are summarized in Fig 1.

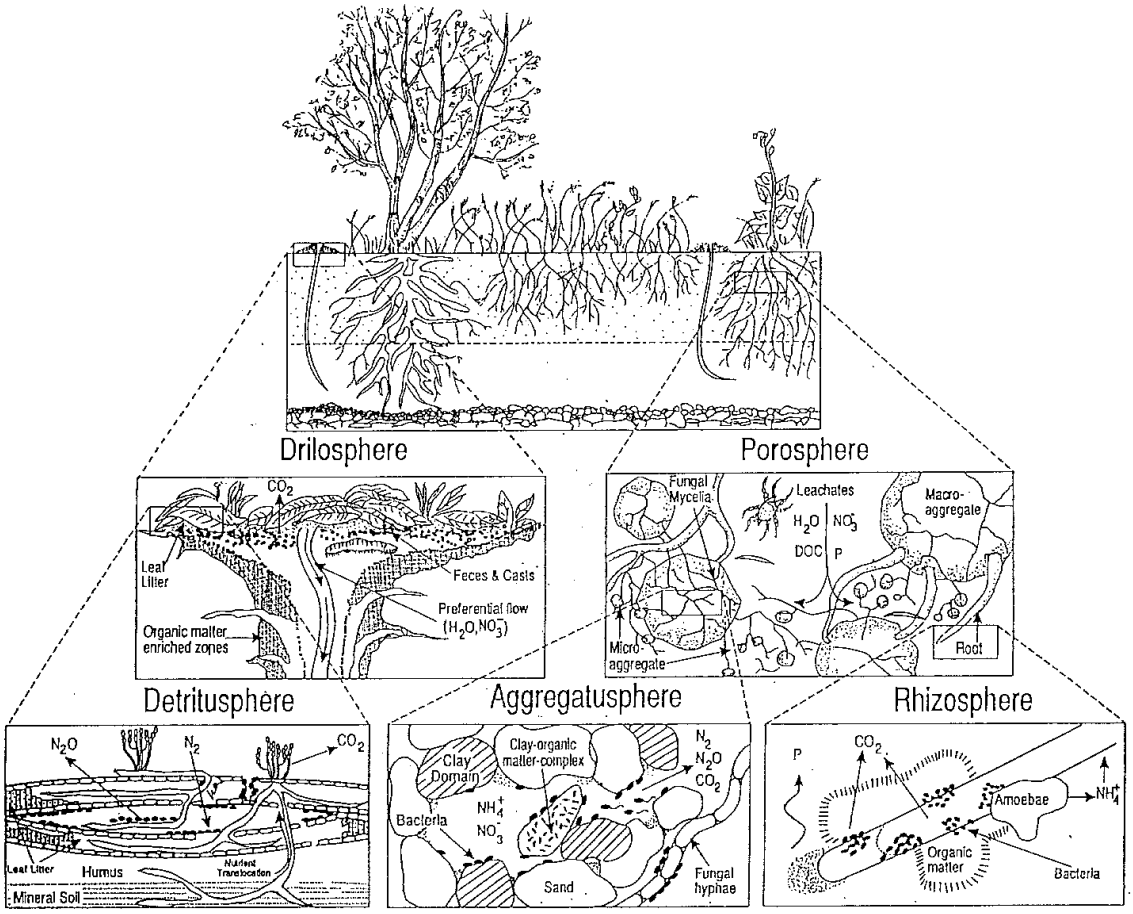


Figure 1: Spheres of influence in the soil being "hot spots" of activity. (Beare et al. 1995)

The **drilosphere** is the fraction of the soil, which has passed through the digestive tract of earthworms. The mean width of the drilosphere is 2 mm according to Bouché (1973) but it can reach between 5 and 10 mm around the burrows of the earthworms *Lumbricus terrestris* in forest environments. This sphere extends in most soil horizons and incorporate and transforms features of the other functional spheres. Thus is not completely independent from them, however it can be distinguished by its origin (Brown et al. 2000).

The **porosphere** is the arrangement of solids and voids with a great variety of sizes, from the nanometer to the centimetre. It is colonized by smaller organisms such as bacteria, protozoa, nematodes (inhabiting water films) and micro-arthropods and mycelial fungi (living in the larger pores) (Beare et al. 1995). Larger soil biota have a major influence on soil porosity and create macropores such as earthworm burrows that form long galleries. Macropores can also

be sites of enhanced microbial activity and are often colonized by microarthropods, nematodes and protozoa (Lee & Foster 1991). When burrows are permanent, they are also preferential sites for root growth (Lee 1985).

The **detritusphere** is the zone where plant and animal detritus undergo decay. This zone has a strong microbial activity due to the presence of decomposers (bacteria and fungi) (Poll et al. 2008). The detritusphere also hosts a part of the soil mesofauna such as small oligochaeta or microarthropods. Alterations of the soil ecosystem such as overpasturing, intensive cultivation and fertilizer applications can reduce micro-organismal diversity through the reduction of microhabitat heterogeneity (Buscot & Varma 2005).

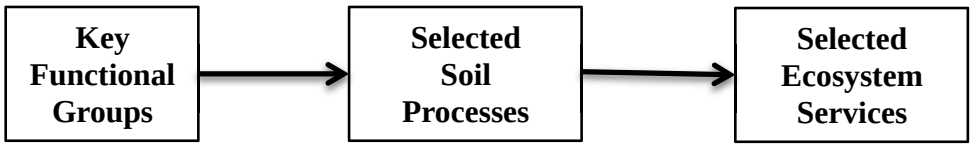
The **aggregatusphere** is formed of many components ranging from clay microstructures and fine particles to micro- (50-250 μm) and macro-aggregates (>250 μm). Carbon and nitrogen cycles in the soil are very dependant on the aggregation of particles in the soil (Brown et al. 2000). It is the main habitat of soil micro-organisms which account for 90% of the soil biomass (Loreau et al. 2002). Microorganisms are the primary agents of aggregate stabilization. Both fungi and bacteria contribute to the formation of soil aggregation through deposition of extracellular polysaccharides and formation of mineral-organic complexes (Gobat et al. 2010).

The zone of influence of roots in the soil is called the **rhizosphere.** It has also been called the "hidden half" because of the important volume roots occupy compared to the volume of the plant (Bowen & Rovira 1991). The compounds produced by live roots stimulate microbial activity and populations, making it a temporally and spatially variable environment (Buscot & Varma 2005). The roots also provide energy as decaying matter when they're dead. They hereby host a great part of the living biomass of the soil. They also host many symbiotic organisms such as mycorrhizal fungi which colonize more than 80% of terrestrial plants (Wang & Qiu 2006).

1.2 Ecosystem services of the soil and the role of soil biodiversity

Ecosystem services are defined as any goods and ecosystem functions that provide benefits to human populations (Lavelle et al. 2006). Ecosystem services in general can be classified into four main categories: those providing resources (food, water-storage and cleansing, combustible), life support services (nutrient cycling, pollination), regulation of different ecosystem functions (climate regulation, disease control, pollution remediation), and cultural services which do not lead to material benefits (recreation, aesthetic and cultural uses) (Barrios 2007). Soil contributes to all these four categories of services provided to mankind: physical and nutritive support for agriculture (including forestry), organic matter and waste degradation, nutrient recycling and regulation of main biogeochemical cycles (carbone, nitrogen, sulphur, phosphorus).

The majority of these services is provided by the activity of soil organisms, however knowledge of their specific contribution is still limited. Soil biodiversity associated with ecosystem services is best considered by identifying groups of organisms that play major roles in ecosystem functioning (Barrios 2007) (Figure 2).



Microsymbionts	Microsymbiosis	Nutrient uptake
Decomposers	Decomposition	Nutrient cycling
Ecosystem engineers	Soil structure modification	Regulation of soil erosion, C sequestration, water flow and storage
Soil-Borne Pests and Diseases	Hervivory, Parasitism	
Microregulators	Predatory, Parasitism	Biological Control of Pests and Diseases
	Grazing of decomposers	Nutrient cycling

Figure 2: key functional groups of soil biota, soil processes they influence and ecosystem services they provide in agricultural landscapes (modified from Barrios (2007).

- Microorganisms realise nearly 90% of chemical transformations of organic substrates in soil (Lavelle et al. 2005). In agriculture the association between plants and nitrogen-fixing bacteria (e.g Rhizobium), as well as plants and arbuscural mycorrhizal fungi are well known cases of positive impacts of microsymbionts on crop yield due to increase in nutrients available for the plant.
- Decomposition of organic materials and transformation into simpler molecules available for plant roots is one of the most important ecosystem process (Barrios 2007). Small invertebrates of the mesofauna and microfauna realise physical fragmentation of organic substrates, increasing surface area and facilitating biochemical degradation via enzymes produced by bacteria, fungi, protozoas and invertebrates.
- Soil macrofauna is mainly represented by earthworms and has a major influence on the soil functioning. Representing the major animal biomass, earthworms cause substantial physical changes in the soil through their burrowing activity. As defined by Jones et al (1994), they actively modify, maintain or create habitats for other

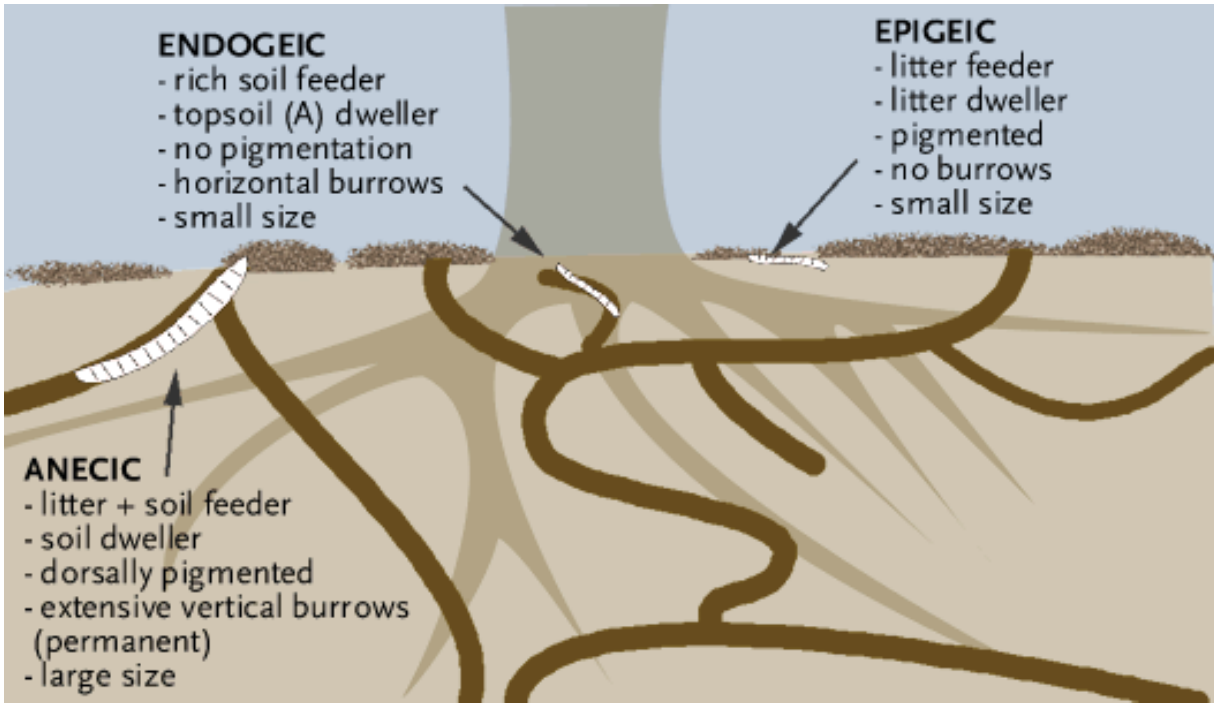
subordinated organisms and are thus characterized as “ecosystem engineers”, hence influencing the whole biological community of soils.

2 The soil lumbricid macrofauna

Earthworms (Annelids, oligochaeta) are the largest component of the soil fauna. In temperate pastures they can be from 600 to 700 individuals per m^2 for a biomass of 1000 $kg.ha^{-1}$. Soils of agrosystems usually have lower densities with 300 to 400 individuals per m^2 and an average biomass of 500 $kg.ha^{-1}$ (Binet 1993; Binet et al. 1997). As early as 1789, Gilbert White had recognized the role of earthworms in the promoters of vegetation, then Savigny in 1826 described their diversity (Lee 1985). However it is the Darwin’s last book “ *The Formation of Vegetable Mould by the Action of Worms with observations on their habits* “(1881) which marked an important step forward and drew attention to the importance of these animals in the processes of nature.

2.1 Elements of earthworm ecology

Earthworms have developed different adaptive strategies based on morphological, behavioural and physiological characteristics allowing them to live in the soil. Bouché (1972) described about 180 species in France and classified them into several ecological categories based on morphological, behavioural, and ecological characteristics described below (Figure 3):



http://www.nrri.umn.edu/worms/identification/ecology_groups.html

Figure 3: characteristics and repartition of the three ecological groups of earthworms

The **Epigeic** worms are small, pigmented earthworms which live in the surface litter. This group is represented by *Lumbricus rubellus*, *Dendrobaena octaedra*, and *Lumbricus castaneus*, among others. They feed upon decaying organic matter present in the litter. They almost do not burrow, but some intermediary species can create small superficial burrows. The epigeic species are the most exposed earthworms to environmental hazards such as predation and cropping operations (soil tillage, pesticides). Hence they are usually absent from cultivated soils.

Endogeic earthworms have various sizes ranging from 1 to 20 cm. They include *Aporrectodea caliginosa* (Figure 4A), *Allolobophora chlorotica* (Figure 4B) or *Allolobophora icterica.* They create burrows with random orientations in the superficial layers of the soil (30 cm). They are geophagic earthworms and feed upon the organic matter already incorporated and mixed with minerals in the soil. Three sub-categories of endogeic earthworms were defined according to the organic matter richness of the soil they inhabit. The *oligohumic*

endogeic worms live in an environment with very limited food resources, deep in the soil and do not move much. The *mesohumic* worms do not select organic particles and live between 10 and 15 cm depth. The *polyhumic* ones ingest selectively organic matter particles and live in the rhizosphere or at the soil-litter interface (Lavelle 1981).

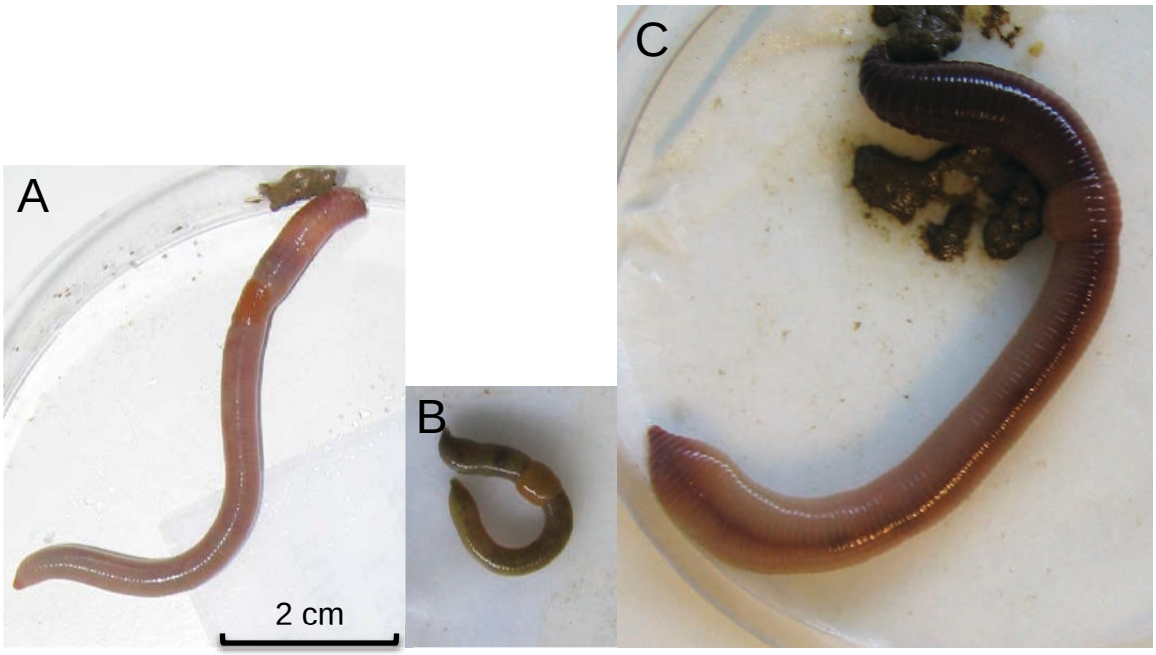


Figure 4: the endogeic earthworms Aporrectodea caliginosa (A) and Allolobophora chlorotica (B) used in the present work, and the anecis species Lumbricus terrestris (C) represented with respect to their respective size (photo: N. Givaudan)

The **anecic** earthworms are usually bigger and pigmented. The group includes *Lumbricus terrestris* (Figure 4C), *Aporrectodea longa or Aporrectodea longa.* They are characterized by strong muscles and a great burrowing activity and some species can reach giant sizes (10 to 110 cm). They feed upon the surface litter mainly during the night and live in long sub-vertical burrows (1 to 6 m) that they create. Through their activity they mix the organic matter in the whole soil column. They are also quite exposed to predation but have a good ability to hide quickly in their burrows in case of danger. It is to be noted that in these species, the "habitat" (the soil) is different from the "food" (the litter).

Earthworms activities are closely dependant on soil temperature, moisture and organic matter , hence most species undergo periods of quiescence or diapause (coiling in small soil cells in

deep soil layers) to escape unsuitable conditions (Edwards & Bohlen 1996). Their peak activity (burrowing and cocoon production) under temperate climates occurs during spring and autumn, hence field sampling mostly take place within these periods.

2.2 The significance of bioturbation by earthworms in ecological processes.

Soil organisms have a major role in soil processes by reworking and physically translocating soil particles when feeding and moving in them. This process is called bioturbation and has firstly been described by Darwin in 1881 although he didn't name it (Wilkinson et al. 2009). In marine sediments, the process of bioturbation is also thought to be a determinant factor explaining the "Cambrian explosion" of life ("burrowing revolution") (Meysman et al. 2006), thus having played a significant role in the evolution of life.

In the soil numerous invertebrates, and not only earthworms, generate bioturbation e.g. termites and ants (Lavelle & Spain 2001), see table 1. Root growth is also contributing to soil bioturbation.

Table 1: Relative importance of bioturbators that mound and mix soil categorised by climate.

Paton et al (1995) cited in Wilkinson et al (2009)

Climate[a]	Ranking
Polar/montane	Vertebrates > ?
Temperate continental	Earthworms > vertebrates ≥ ants > other invertebrates
Temperate maritime	Earthworms > ants > vertebrates
Mediterranean	Earthworms > vertebrates > termites ≥ ants
Semi-arid	Vertebrates > termites ≥ ants
Humid subtropics	Ants = earthworms = vertebrates > termites
Tropical wet and dry	Earthworms > termites = ants
Humid tropics	Earthworms > termites
Arid	Vertebrates > invertebrates

From an ecological vision, bioturbation by organisms is recognized as efficient ecosystem engineering, and their presence or absence has a tremendous impact on the ecosystem (Jones

et al. 1997). Bioturbation has an effect on the sediment texture and structure, and the dispersal of solid particles as well as of soil microorganisms, thus it plays a major role in the ecosystem functioning. It is recognized that up to 50% of aggregates (>250µm) in the soil are earthworm casts (Lee 1985). In a soil where a diverse lumbricid community is established, the water retention capacity and the aggregate stability are increased, the void space is bigger, and the water infiltration rate is higher than in a soil without earthworms or with only surface-dwelling species (Anderson 1988). Anecic species provide vertical channels for water infiltration and gas exchange, whereas endogeic earthworms provide more horizontally oriented, frequently extensive and intersecting networks of macropores that promote water movement and gas diffusion.

Earthworm bioturbation also has a major role on the regulation of soil microbial activity. Bioturbation creates soil heterogeneity and thus sustain specific niches for microorganisms (microbial "hotspots") (Binet et al. 1998; Monard et al. 2008). It was also shown to increase the diversity of atrazine-degrading bacteria in soils and enhance the degradation of the herbicide (Monard et al. 2011).

2.3 Earthworm communities and agriculture

The beneficial impact of earthworms on agroecosystems is part of a feedback loop, because the agricultural practices influence in turn the earthworm communities. In agroecosystems, the intensification of human activities such as cultivation operation, crop rotations, mineral fertilization, and pesticide applications tend to degrade biological and structural properties of the soil as well as reduce earthworm populations (Binet 1993; Edwards & Bohlen 1996). Each of these four main human inputs interacts strongly with each other in a specified management system.

- **Tillage**

Darwin called earthworms "nature's plough" but he did not focus on how human ploughing affected earthworm communities. It is now well established that soils being subjected to

tillage or ploughing host smaller earthworm communities than untilled soils or grasslands in most parts of the world (Jordan et al. 2004; Smith et al. 2008).

The decrease in earthworm number and diversity is believed to be due to mechanical damage, to the loss of protecting vegetation cover, to decreased supply of food, but also by increased predation when worms are brought to the surface (Edwards & Bohlen 1996). However the practice of replacing cultivation operations by chemically erasing the crop residue and weeds by herbicides attracts interest and is adopted by farmers throughout the world. With this practice the activities of earthworms are preserved and are becoming increasingly more important in removing organic matter from the soil surface and maintaining soil fertility (Edwards & Lofty 1982). Because of their size, the deep-burrowing species such as *Lumbricus terrestris* or *A. longa* usually benefit more from the reduction in tillage, than the smaller endogeic earthworms (Edwards & Lofty 1982; Nuutinen 1992; Edwards & Bohlen 1996). Emmerling (2001) showed that abundance and fresh biomass of the total earthworm community, and the number of individuals of the dominant species *A. caliginosa* were enhanced by layer cultivation (conservation tillage) compared to regular ploughing management (Figure 5).

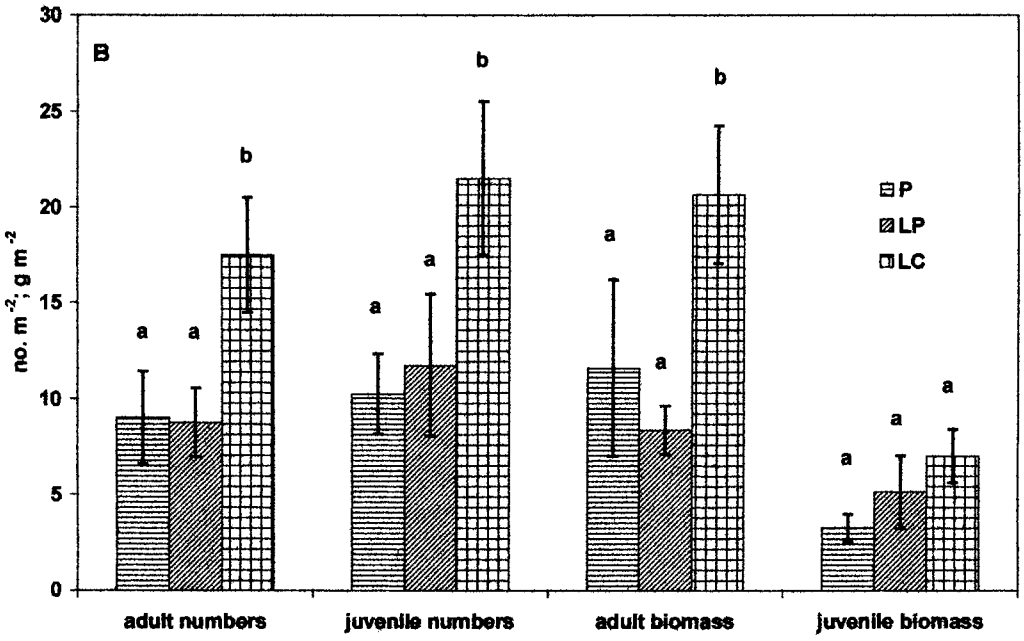


Figure 5 : Mean (±S.D., n = 16) abundance and fresh biomass of adult and juvenile earthworms under various tillage techniques in plots with winter rye from an experimental field at "Eichenhof", Rommersheim, Germany, from 1995 to 1998. Different letters indicate significant differences between treatments for each earthworm category, P < 0.05, Tukey-B-test (P: ploughing; LP: two-layer ploughing; LC: layer cultivation). (Emmerling 2001).

- **Cropping**

It is likely that the most important factor in crop rotations and types of culture influencing earthworm communities and numbers is the amount of organic matter available as food for earthworms (Edwards & Bohlen 1996). Due to the deleterious effects of tillage mentioned in the previous paragraph, annual crops usually have deep detrimental impacts because of the rotations themselves, and thus tillage operations (Decaëns & Jiménez 2002). Nevertheless cropping can have an influence on the number of earthworms. Edwards and Bohlen mentioned several studies showing that some types of crop rotations favoured earthworms' numbers. In particular the number of animals were lower under row crops and greater in plots growing winter cereal and summer legumes (Hopp 1946). A more recent study by Haynes and co-workers (Haynes et al. 2003) pointed that sugarcane was more beneficial to earthworm communities than maize cultivation in South Africa. The rotation pea/winter wheat/linen/wheat (I) had a higher earthworm density compared to sugar beet/winter wheat/maize/winter wheat (II and III have similar crop rotations but with increasing soil compaction, Table 2 Capowiez *et al* (2009)). In general the inclusion of cereals that leave

considerable residues promotes earthworm populations. Perennial crops such as alfalfa or clover are also particularly beneficial to earthworm populations because of the absence of tillage and also the high protein content of the cultivation residues. Root crops, as the whole plant is removed, tend to decrease earthworm populations (Edwards & Bohlen 1996).

- **Fertilizers:**

Fertilization can have different impacts depending on the product applied e.g organic or inorganic amendment. The effects of the fertilizers can be direct, e.g. by modifying the soil conditions (pH), by serving as direct food source (in case of organic fertilization) for surface-feeding earthworms (i.e anecic species), or indirect, by influencing the vegetation which can ultimately turn into decaying matter and thus food for the animals (Edwards & Bohlen 1996; Whalen et al. 1998). Whalen *et al* (1998) reported that earthworm abundance and biomass were significantly greater in manure-amended plots compared to plots treated with inorganic fertilizers (Figure 6). In forage corn crops, Mijangos *et al* (2006) showed that organic fertilization and no-tillage management increased earthworm abundance by 226.9% than in inorganically fertilized, conventional tillage plots along with several other beneficial biological parameters. However here the authors did not separate the effects of tillage from the fertilization.

Table 2 : mean values and species relative abundance, total biomass and percentage of juveniles depending on two tillage managements (CT for conventional tillage, RT for reduced tillage) and three cropping systems with increasing soil compaction (I, II and III). The two factors were compared separately, and significant differences between each modality were indicated by different letters (Capowiez et al. 2009).

	Tillage management		Cropping system		
	CT	RT	I	II	III
Abundance (no. m^{-2})	110.6^{a}	116.2^{a}	122.7^{A}	122.1^{AB}	95.6^{B}
L. terrestris (no. m^{-2})	10.1^{b}	22.5^{a}	14.0^{A}	19.7^{A}	15.2^{A}
A. giardi (no. m^{-2})	2.2^{b}	27^{a}	6.1^{B}	20.8^{A}	16.8^{A}
A. caliginosa (no. m^{-2})	54.2^{a}	23.9^{b}	56.5^{A}	31.3^{B}	29.5^{B}
A. rosea (no. m^{-2})	16.6^{a}	19.6^{a}	14.4^{A}	24.3^{A}	15.6^{A}
Biomass (g m^{-2})	36.9^{b}	76.8^{a}	46.9^{B}	62.4^{A}	61.1^{A}
Percentage of juveniles	51.03^{a}	49.29^{a}	47.78^{A}	53.35^{A}	49.44^{A}

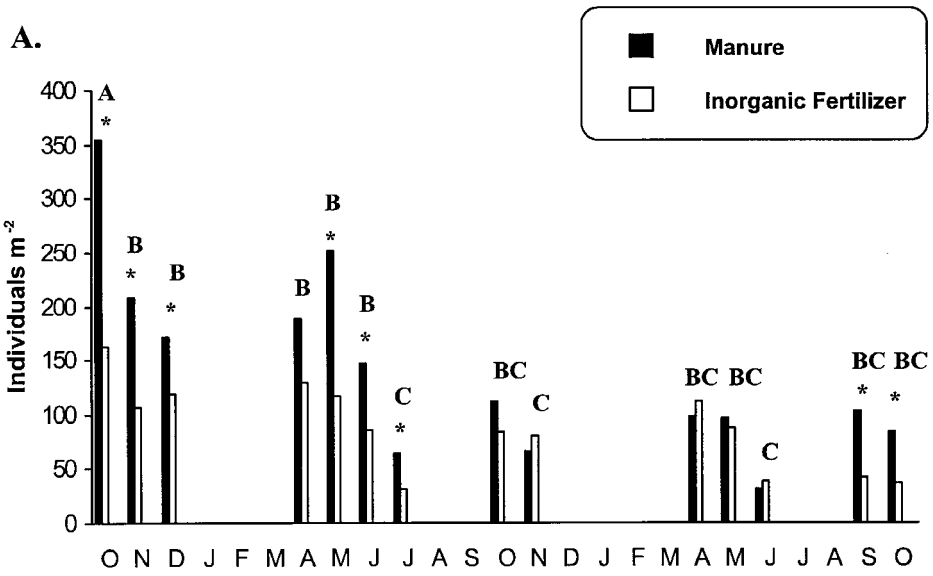


Figure 6: seasonal fluctuations in mean monthly total earthworm community abundance in corn agrosystems receiving two different fertilization treatments from 1994 to 1996. Asterisks indicate significant differences between fertilization treatments. (Whalen et al. 1998).

Some species are dominant in agrosystems, such as *Aporrectodea caliginosa* where others are usually not present (i.e. epigeic species in general) (Nuutinen 1992; Söchtig & Larink 1992; Lamandé et al. 2003; Jordan et al. 2004). It is likely that these species maintain their numbers in cropped soils through adaptation to agricultural practices. Impacts of land use on earthworm communities are likely to have significant consequences on soil functioning especially if functional groups of organisms disappear such as specific ecological groups of earthworms (e.g. the loss or reduction of endogeic species) (Walker 1995; Decaëns & Jiménez 2002; Mori et al. 2013). Eventually the soil ecosystem resilience to anthropogenic perturbations is probably partly dependant on earthworm tolerance to agricultural practices.

3 Ecological risk assessment of pesticides in soils

The use of pesticides in agriculture takes on two paradoxical aspects having totally opposed consequences. The first aspect deals with the necessary reduction of the damage caused to crops and their yields by pest organisms (crop-eating animals, crop infecting fungi and

bacteria, and weeds) in order to achieve good agricultural productivity. The economic consequences of crop destruction by pests can be drastic for a farm or a whole country. The second aspect sits on the nature of the pesticides themselves, which makes them, under certain conditions, pollutants of the air, water, soils and food products because of their toxicity against pests but also for non-target organisms (Calvet et al. 2005).

3.1 Actual pesticide uses – Europe, France

Pesticides, according to their usage (i.e pest types), have a broad range of chemical natures and modes of action (Table 3).

Table 3 : a non-exhaustive classification of pesticides by target, mode of action and chemical structure (Arias-Estévez et al. 2008)

Classifications of pesticides

By target		By mode or time of action		By chemical structure
Type	Target	Type	Action	
Bactericide (sanitizers or disinfectants)	Bacteria	Contact	Kills by contact with pest	Pesticides can be either organic or inorganic chemicals. Most of today's pesticides are organic
Defoliant[a]	Crop foliage	Eradicant	Effective after infection by pathogen	
Desiccant[a]	Crop foliage	Fumigants	Enters pest as a gas	
Fungicide	Fungi	Nonselective	Toxic to both crop and weed	
Herbicide	Weeds	Post-emergence	Effective when applied after crop or weed emergence	Commonly used inorganic pesticides include copper-based fungicides, lime-sulfur used to control fungi and mites, boric acid used for cockroach control, and ammonium sulfamate herbicides
Insecticide	Insects	Pre-emergence	Effective when applied after planting and before crop or weed emergence	
Miticide (acaricide)	Mites and ticks	Preplant	Effective when applied prior to planting	
Molluscicide	Slugs and snails	Protectants	Effective when applied before pathogen infects plant	
Nematicide	Nematodes	Selective	Toxic only to weed	Organic insecticides can either be natural (usually extracted from plants or bacteria) or synthetic. Most pesticides used today are synthetic organic chemicals. They can be grouped into chemical families based on their structure
Plant growth regulator[a]	Crop growth processes	Soil sterilant	Toxic to all vegetation	
Rodenticide	Rodents	Stomach poison	Kills animal pests after ingestion	
Wood preservative	Wood-destroying organisms	Systemic	Transported through crop or pest following absorption	

[a] In U.S. law the term "pesticide" is defined to cover not only pesticides proper, but also these other classes of agrochemical.

In the European Union, the use of pesticides has increased steadily above 200000 tons of active ingredient since the beginning of the 1990s, then remained relatively constant until 2002, followed by a decrease in 2003 (figure 7, Eurostat, European Commission 2007).

The total decrease since the end of the 90s is apparently due to the reduction in fungicides as they form the majority of the pesticides used (Figure 7). The withdrawal of some products used in large amounts, and their replacement by products used at lower dosage rates could also explain the decrease observed since the end of the 90s.

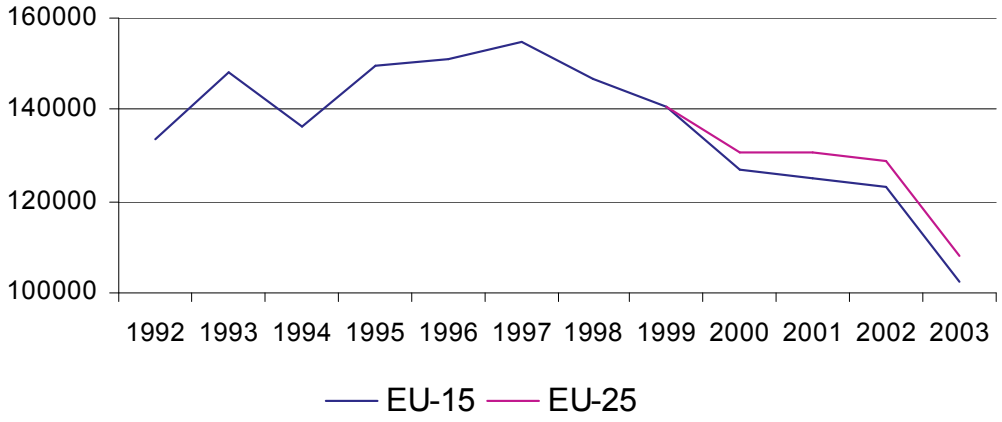


Figure 7 : use of fungicides by EU member States in tons of active substance (EU-15 and EU-25), 1992-2003 (Eurostat, European Commission 2007)

Five countries account for almost 75% of the pesticides used in Europe, because of their size and intense agricultural activities. France alone uses 28%, Spain and Italy 14 % each, Germany 11% and the United Kingdom 7%. France, Italy and Spain use 64 % of the total amounts of fungicides, mainly sulphur inorganic fungicides used on grape production.

Herbicide consumption is the highest in France, Germany and Spain, due to cereals and maize production. In France, the amounts of pesticides used increased from 1992 and peaked in 2000, then decreased from 2000 to 2003. The two main categories of pesticides used are fungicides and herbicides and showed a similar overall reduction between 2000 and 2003 (Figure 8):

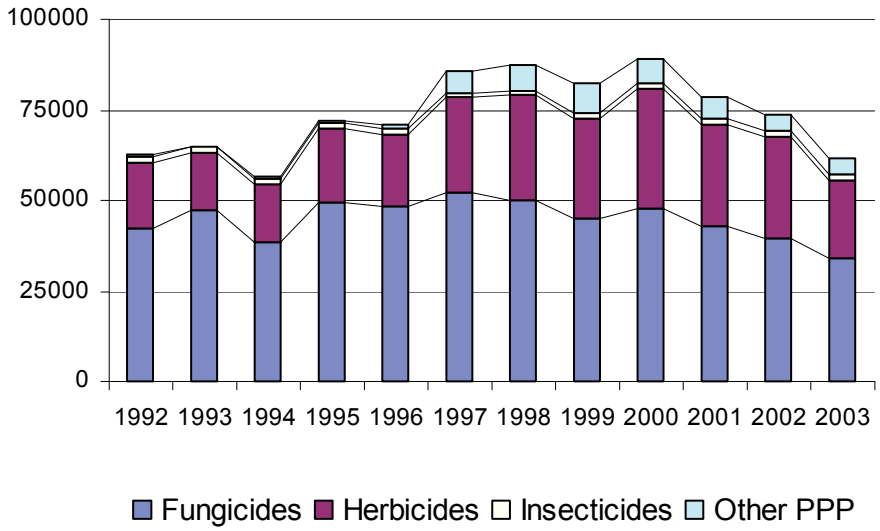


Figure 8 pesticides (or Plant Protection Products –PPP) used in France 1992-2003, in tons of active substance (Eurostat, European Commission 2007).

The three main pesticide-using crops in 2003 (as measured by tonnes of active substance) are grapes, followed by cereals and maize (Figure 9).

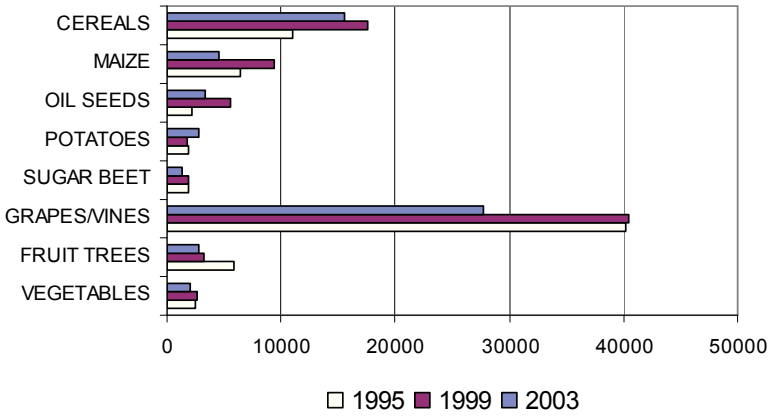


Figure 9 quantity of pesticides used by crop type in tonnes of active substance in France (Eurostat, European Commission 2007).

The figure 10 describes the main herbicide and fungicide active ingredients used in percentage of treated crops. In 2006 in France, epoxiconazole was the first fungicide molecule and glyphosate the sixth herbicide molecule used on arable crops in percentage of treated areas.

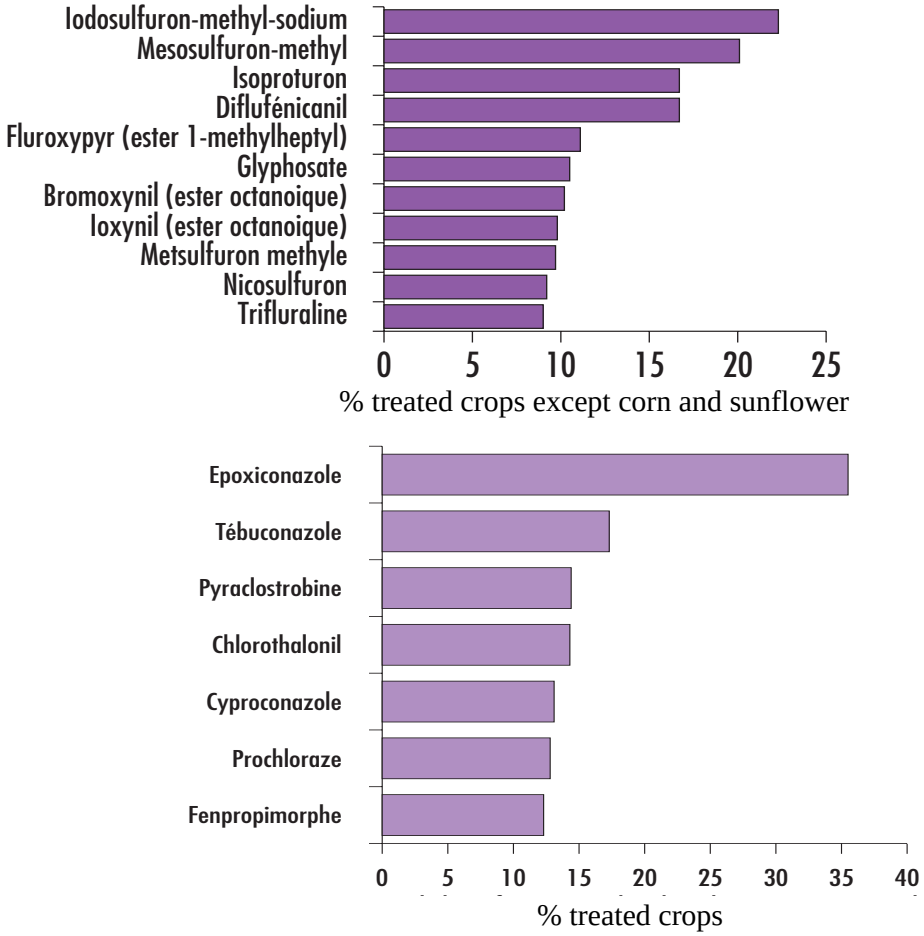


Figure 10 : main active molecules used as herbicides (top) and fungicides (bottom) in % of treated areas (Ministère de l'Alimentation de l'Agriculture et de la Pèche 2010).

3.2 Pesticide dynamics in soils

3.2.1 Pesticide losses from pesticide applications

When applied on crops the pesticide can follow several pathways before and after reaching the crop, leading to major losses (Figure 11).

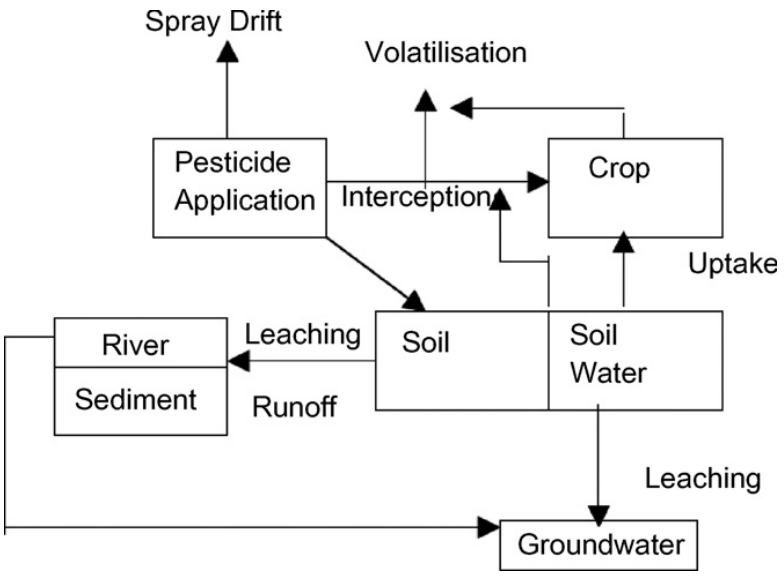


Figure 11 : Pathways of a pesticide applied to a crop (Arias-Estévez et al. 2008)

In fact, only a very small part of pesticide is actually deposited on the target insect or plant. The proportion of pesticide reaching its target depends very much on the target pest and the mode of application. An example of calculation of pesticide consumed by the caterpillars *Pieris rapae* showed that 0.003 % of the 1 kg/ha of pesticide applied was actually received by the target animals (Pimentel & Levitan 1986). The losses can also be particularly high when insecticides are applied to control flying insects.

The proportion of fungicides actually consumed by target plant pathogens is likely to be even lower than for insecticides, because the fungal targets are small. The amount of herbicides reaching target plants is on the other hand usually higher. According to Pimentel and Levitan (1986) it can reach 0.1 to 0.5% for post-emergence herbicides applied on corn fields, and up to 80% if it is directly applied to a weed tree. Because of these losses, a high amount of pesticide must be applied to ensure sufficient contact and concentration at the target pest or plant. Eventually, the soil system is the primary sink for many pesticides (Xia & Leidy 2002). When the pesticide has been applied, there are three main routes by which they can enter the soil: spray drift to soil during application, wash-off from treated foliage, and release from granulates applied to the soil (Arias-Estévez et al. 2008).

3.2.2 Formation of bound residues in the soil and bioavailability to earthworms

When they end up in the soil system, pesticides can bind to organic or mineral particles and thus become non-extractable by most solvents. These bound residues have been defined in different ways, but a generally accepted definition is the one put forward by Robert (1984) and modified by Fuhr *et al* (1998) to include reference to the structure of the matrix. Calderbank *et al* (1989) also emphasized that the bioavailability of bound residues was actually the most important matter, rather than their definition, which was added to the definition (Führ et al. 1998):

"Bound residues represent compounds in soil, plant or animal which persist in the matrix in the form of the parent substance or its metabolite(s) after extractions. The extraction method must not substantially change the compounds themselves or the structure of the matrix. The nature of the bond can be clarified in part by matrix-altering extraction methods and sophisticated analytical techniques. To date, for example, covalent ionic and sorptive bonds as well as entrapments have been identified in this way. In general the formation of bound residues reduces the bioaccessibility and the bioavailability significantly."

Mordaunt *et al (2005)* compared the extraction efficiency and the proportion of non-extractable fraction of six common pesticide molecules (atrazine, dicamba, isoproturon, lindane, paraquat, and trifuralin). The authors used several solvents with increasing strength to test the bond of the pesticides to the soil matrix. Soil was sampled from an agricultural research station without any treatment (i.e sterilisation or removal of organisms), and the pesticide was added by diluting it in an acetone-methanol mixture. They determined the different fractions of the pesticides bound to the soil as "soil water" fraction (readily available), "organic extractable fraction" (potentially available) and "non-extracted" fraction. Moreover, the amount of pesticide that underwent degradation was also monitored by

trapping CO_2 to complete the mass balance. The results are presented in Figure 12:

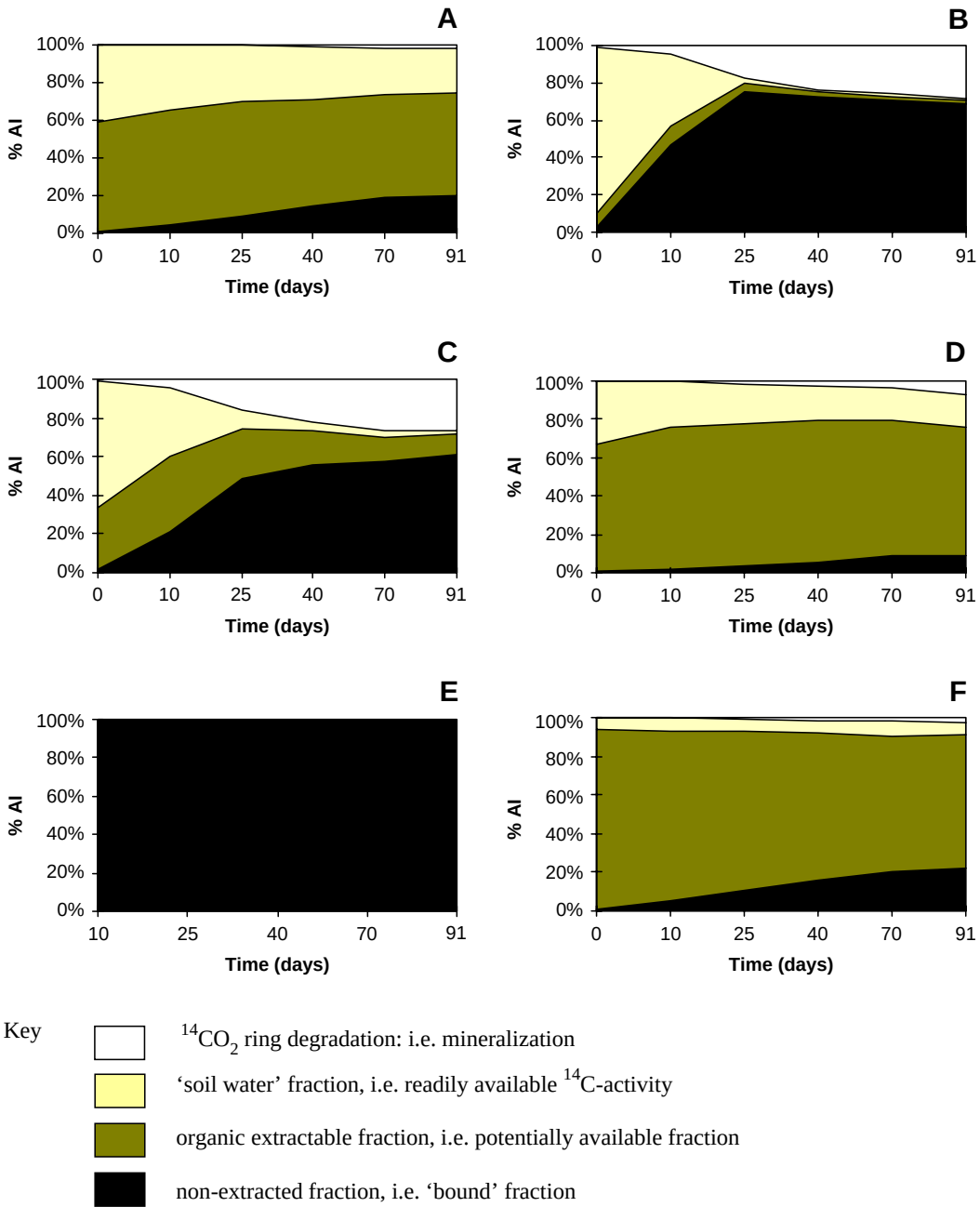


Figure 12 : the fractions extracted from soil by different solvents of atrazine (A), dicamba (B), Isoproturon (C), Lindane (D), Paraquat (E), and Trifluralin (F).

They defined three types of behaviour:

- Type A is represented by atrazine, lindane, and trifluralin (A, D, F on the figure). The main portion of the pesticide is extractable (80-90%), the majority of which by organic solvent (i.e. “strong” extraction) and a smaller part by “light” extraction with water. They undergo relatively little degradation (2-8% after 91 days).
- Type B behaviour is expressed by Dicamba and Isoproturon (B and C on the figure). They undergo more rapid degradation (around 25% after 91 days). At the beginning a large proportion (90-100%) of the molecule is extractable, but the amount of “bound” residues increases fast and reaches rapidly 55-65%.
- Type C behaviour is exhibited by paraquat (E on the figure). Most of the compound is quickly and tightly held to the matrix.

The same authors tested bioavailability of “bound” residues (i.e. aged) compared to “fresh” residues (i.e. freshly introduced) of the same two type B (dicamba and isoproturon) and atrazine as type A pesticides to the earthworm *Aporrectodea longa* (Gevao et al. 2001). Earthworms bioaccumulated 2-10 times more of the compounds when it was freshly introduced compared to previously bound to the soil. Moreover, the introduction of earthworms seemed to retard the formation of bound residues (Gevao et al. 2001). However, Binet *et al* (2006) and Fahrenhorst *et al* (2000) found opposite results, bioturbation by earthworms of a soil contaminated by atrazine increased the proportion of bound residues.

After prolonged aging of pesticide residues in soil, pesticide molecules can undergo sequestration (entrapment) in structural voids and hydrophobic interiors of micelle-like humic aggregates or in micropores of organic matter and clays (Gevao et al. 2001). This phenomena can decrease the toxicity of pesticides to soil organisms by rendering the molecule less bioavailable. The formation of bound residues can thereby be seen as positive as it immobilizes pollutants in the soil and decreases their impacts because of natural attenuation of pesticide toxicity. On the other hand they constitute a “hidden fraction” (Gevao et al. 2000) that can potentially be released slowly and contaminate soil as well as ground and surface waters (Johnson et al. 1999; Gevao et al. 2003). There is a need for a better understanding of

this phenomenon and its possible implications in the “residual” contamination of ground and surface waters by pesticides that are now banned for a long time, for instance atrazine.

3.2.3 Influence of earthworm activity on pesticide fate

Several studies have reported the impact of soil macrofauna on pesticide fate in the soil.

As stated above in 3.2.2, it is still not clear whether earthworms delay or increase the formation of pesticide bound residues, as contradictory results have been shown in different contexts. However, earthworms have a great impact on the soil microbial community by comminution, burrowing and casting, grazing or dispersal (Brown 1995; Scheu et al. 2002). In particular, recent studies have shown that they can influence the soil pesticide degraders thereby enhancing pesticide degradation. Monard *et al* (2011) evidenced that earthworm bioturbation enhanced the atrazine degrader diversity of the soil microflora by generating heterogeneity in the soil and increased the pesticide degradation rate. Alekseeva *et al* (2006) demonstrated that atrazine adsorption was greater on earthworm casts than on bulk soil, and this was due to non-decomposed organic matter and particular compounds present in the casts. It was proposed that specific hydrophobic interactions occur between Atrazine and soil organic matter in the earthworms’ casts. Liu *et al* (2011) also showed that the earthworm *Aporrectodea caliginosa* stimulated the degradation rate of the herbicide 2-methyl-4-chlorophenoxyacetic acid (MCPA), and stimulated abundance and activity of the herbicide’s microbial degraders. Schreck *et al* (2008) showed that the concentrations of a mixture of two insecticides and four fungicides decreased faster after 34 days with the presence of earthworms than without, but not by bioaccumulation in the animals’ tissues.

On the one hand, there is a need to better understand the implication of earthworms in the formation (enhancement or delay?) of bound residues. On the other hand, more knowledge into the role of earthworms in pesticide degradation is needed, as only a few studies have specifically addressed this subject.

As stated before, a large part of the applied pesticides do not reach their target and end up in the soil. The pesticide residues may impact in return earthworm populations via acute or chronic toxicity, and for the last three decades, this has led to a considerable scientific production (Roberts & Dorough 1985; Greig-Smith et al. 1992; Bouché 1992; Seiber & Ragsdale 1999).

3.3 Toxicity assessment of chemicals to lumbricids and regulatory aspects

3.3.1 Pesticides impacts on earthworms

Pesticides can affect earthworms at several levels of biological organization, starting from sub-organismal i.e physiological level, with consequences on higher levels such as individual and population levels (Figure 13). A comprehensive investigation of the effects of a pollutant at all these levels leads to Ecological Risk Assessment (ERA).

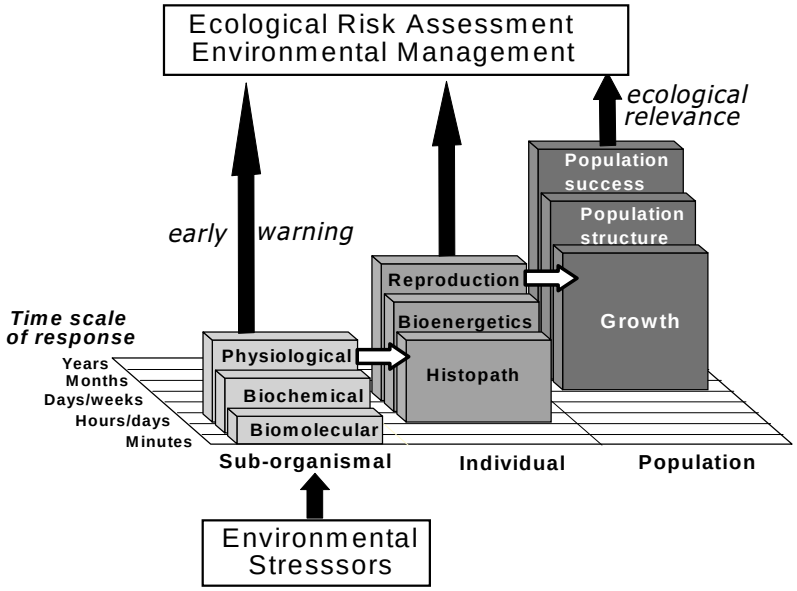


Figure 13: Time and scale of response of organisms to environmental stressors at the different levels of biological organization (Adams & Greeley 2000).

In the soil, earthworms' exposure to xenobiotics including pesticides can occur directly via uptake from interstitial water (porewater) through the outer skin and from ingestion of contaminated soil particles through gut epidermis (Belfroid et al. 1993; Belfroid et al. 1996).

Characteristics of the soil impact the toxicity of pollutants via soil binding capacity (CEC, organic matter and pH), hence bioavailability, or also by modifying earthworm behaviour, e.g reduced activity and litter-feeding (Ma 1984; Van Gestel 1992).

Pesticides effects on earthworms can be measured from the laboratory to field scale i.e. with increasing ecological relevance, which is generally associated with less precision in the response (Sanchez 2007). The amount of the pesticide applied is of great importance. Toxicity can be measured as acute (i.e. high dose, short exposure) or chronic (low dose, long-term) studies. A number of indicators based on medical drugs science have been developed and are nowadays used in reglementary tests (see 3.3.2) such as LC_{50} (lethal concentration for 50% of the individuals), EC_{50} (Effective concentration for 50% of the individuals) or NOEC (No Observed Effect Concentration). In studies made before the 1980s, mortality, assessed by acute toxicity tests (LC_{50}) usually beyond ecologically relevant concentrations was the main parameter measured. Some old examples of very toxic (acute toxicity) chemicals to earthworms include cholinesterase-inhibiting insecticides such as aldicarb and carbofuran (Stenersen 1979). The toxicities of these chemicals eventually led to their interdiction by the EU by non-renewing of their market authorisation (91/414/EEC Council Directive concerning the placing of plant protection products on the market).

However it is generally agreed that sub-lethal impacts, such as reproduction or growth impairment, occur at concentrations much lower than LC_{50} (Pelosi et al. 2013). Van Gestel *et al* (1992) compared lethal (as measured by LC_{50}) and sub-lethal (NOEC) toxicity in aquatic and terrestrial toxicology. He pointed out that in both aquatic and terrestrial systems, there is a similar great variability in the chemicals' lethal and sub-lethal toxicity as measured by the ratios LC_{50}/NOEC: the ratios ranged from 1.7 to 450 in a study with three fish species (Adema 1981), and from 2 to 100 in the study by Van Gestel *et al* (1992). Pelosi *et al* (2013) suggested that, contrary to aquatic toxicology and according to the study by Van Gestel *et al* (1992) the NOEC in terrestrial toxicology is often much lower than the LC_{50}, meaning that

sub-lethal impacts happen at much lower concentrations than the ones expected to cause mortalities.

Hence, there is a gap of knowledge to fill in the comparison of these differences between lethal and sub-lethal toxicity in terrestrial toxicology to understand the inherent differences in bioavailability, uptake and modes of action compared to aquatic toxicology.

3.3.2 Earthworms as model species in ecotoxicology

When it became apparent that earthworm toxicity studies were necessary to provide environmental assessments of chemicals, the need for an easy-to-rear species to standardize ecotoxicological tests appeared. Böstrom and Lofs-Holmin concluded in 1982 that "… the number of methods used until now equals the number of papers presented on the subject". The well known "compost worm" epigeic species *Eisenia fetida* was then chosen in order to provide repeatable, standardized results that could be used for regulatory purposes (Edwards 1984; OECD 1984). Nowadays, mortality, reproduction, and avoidance tests on *Eisenia fetida* are realized prior to authorization of chemicals (OECD 1984; ISO 11268-1 1993; ISO 11268-2 1998; ISO 17512-1 2008)

However Bouché and other scientists advised that this species was recognized phylogenetically heterogeneous and was in fact a "complex" of species, thus responses measured were likely not to be repeatable between different locations (Bouché 1992). Furthermore, when monitoring the effects of agricultural pesticides, the use of this epigeic species was criticized as being not "ecologically relevant" as it is usually absent from agricultural fields (Dittbrenner et al. 2010). With a growing number of studies using this species and comparing it with others, it appeared that it was also less sensitive to environmental toxicants than other earthworm species.

This aspect was recently assessed by a meta-analysis by Pelosi *et al* (2013). The study, using LC_{50} values in 44 experimental treatments, showed that LC_{50} values were on average significantly lower in *Lumbricus terrestris* and *Aporrectodea caliginosa* than for *Eisenia*

fetida. The authors therefore advise to use *A. caliginosa* as model species in the future for pesticide homologation tests.

When conducting ecotoxicological studies, a common practice applied in most cases is to sample individuals from a "pristine area", i.e. an area with no recent contamination by pollutants. This practice underlines the assumption that the model animals, if sampled from a polluted area, can be tolerant to the molecule, either via physiological (pre-exposure) or genetic (long-term exposure) adaptation. It clearly shows that these adaptive reactions (adaptations or acclimatizations) are not sufficiently understood.

4 Adaptive strategies to pollution

4.1 Adaptation or acclimation? Genetic and physiological processes

In ecology, p*hysiological adaptation* e.g. *acclimation* is the expression of the ecological plasticity of species. It is an acquired phenotypical modification that is limited in time, sometimes up to the lifetime of the organism. For example, when Antarctic springtails are taken from "summer" temperatures (5°C) to lower temperatures of -2° via a 2°C acclimatisation step, they show a marked drop at the temperature at which they freeze, compared to non-acclimatized ones. The experiment indicates that the acclimatization process has induced changes in their metabolism, giving them a better capability to cope with the cold (Townsend et al. 2008).

Adaptation is another stage, when adjustments to environmental variability are genetically transmissible to the offspring (Ramade 1984), one of the furthest steps of it being the development of ecotypes, due to geographical variations within species. Genetic adaptation can result from selection pressure by a toxicant in a polluted environment (Weis & Weis 1989).

So far, the best-known cases of resistance to pesticides have been shown in insect pest populations, which are directly targeted by the pesticides, among which organochlorines, pyrethroids, organophosphate and carbamate insecticides. It is considered to be one of the major obstacles to the control of medically and agriculturally significant arthropod pests (Scott et al. 1998; Scott 1999). These resistances generally appear after repeated applications of pesticides that are initially lethal to never-exposed ("naïve") animals, and selection pressure acts quickly, to produce several inherited resistant traits. Common examples of insecticide-resistant terrestrial pest populations are in house flies (*Musca domestica)*, drosophilas (*drosohila melanogaster)*, lepidopteras such as tobacco budworms (H*eliothis virescens and Helicoverpa armigera*) and mosquitoes (*Culex spp.)* (Scott 1999).

Acclimatisation and adaptation also occur in non-target organisms exposed to contaminants, which is the subject of the present work. Due to their inherent persistent nature, adaptation to heavy metals in particular have been the subject of many studies including earthworms (Posthuma & Van Straalen 1993; Donker et al. 1993; Fisker et al. 2011; Fisker et al. 2013). But so far, no study has attempted to investigate acclimatisation or adaptation of earthworms to organic pollutants such as pesticides.

Biota living in contact with the soil can experience toxic effects at several levels of biological organization, i.e. from the sub-individual to the population and community levels. Investigating the development of tolerance proceeds also by considering these different levels of organization. Because of the physiological or genetic changes of adaptation, it is expected that contaminant-adapted population will suffer "costs" (or trade-offs). These "costs of tolerance" are expected to be mainly due to the additional energy necessary to cope with high burdens of pollutants, leaving less resource available for other processes (Posthuma & Van Straalen 1993; Fisker et al. 2011). In plants, this argument is supported by the usual absence of metal tolerant plant populations, less competitive, nearby untolerant ones despite evidence for considerable gene-flow (Posthuma & Van Straalen 1993). 20 years ago, Posthuma and Van Straalen (1993) pointed in a mini-review that considerable gaps of knowledge existed about "costs of tolerance", i.e. consequences for fitness of contaminant-adapted populations.

4.2 Adaptation responses at sub-individual levels

Exposure to xenobiotics in animals often induces or enhances the synthesis of different groups of protective reactions. Many of them have been used as markers of contamination of organisms and were thus called “biomarkers” (NRC-National Research Council 1987). Those involved in adaptation or resistance to xenobiotics will be briefly introduced.

In animals, biodegradable chemicals may be detoxified via several mechanism classified as phase I (alteration of the parent compound by oxidation, reduction or hydrolysis), phase II (conjugation to more polar groups of the body e.g glutathione), and phase III enzymes (catabolisation or direct excretion of conjugated metabolites). Other mechanisms playing a role in the protection against xenobiotics will be mentioned here as they can have a function in adaptive processes e.g heat-shock proteins and antioxidant enzymes.

One of the major group of biotransformation enzymes involved in the phase I metabolism of xenobiotics are cytochrome p450 monooxygenases. The CYP gene superfamily consists in a large number of genes encoding for p450 enzymes. They typically catalyse mono-oxygenations reactions (oxidation, dealkylation, epoxidation or hydroxylation) of a wide range of xenobiotics (Ortiz de Montellano 1986; Guengerich 1987). Daborn *et al* (2002) showed that overtranscription of a single cytochrome p450 gene (Cyp6g1) was responsible for insecticide resistance in populations of *Drosophila melanogaster*. Interestingly, Lukkari *et al* (2004) demonstrated that exposure to metals (Cu/Zn) in the earthworm *Aporrectodea tuberculata* in the laboratory increased the activity of the cytochrome p450 enzyme, and that it was enhanced by a pre-exposure to high levels of metals in the field. Hence the authors showed a possible mechanism of acclimation to metals via p450 enzymes (fig 15).

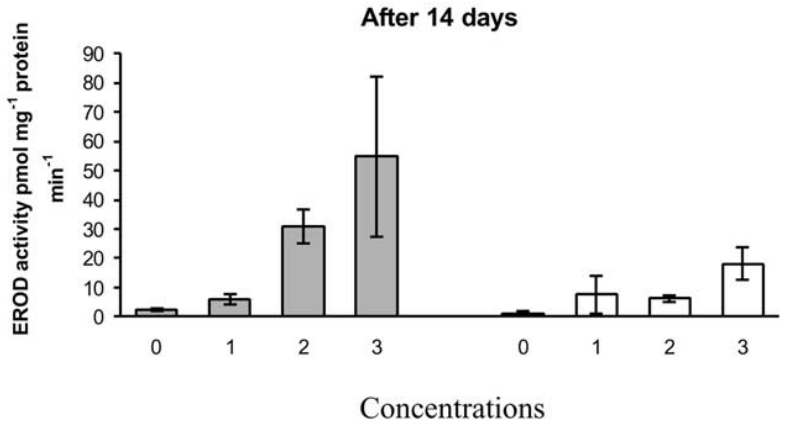


Figure 14 microsomal cytochrome p450 (CYP1A) measured with ethoxyresorufin-O-deethylase (EROD), activity (pmol mg^{-1} protein min^{-1}) in two populations of the earthworm Aporrectodea tuberculata with (hatched bars) and without (white bars) earlier metal exposure from the laboratory experiment after 14 days exposure to different Cu/Zn concentrations (0=control, 1=100/175, 2=200/350, 3=400/700 mg Cu/Zn kg^{-1} dry weight of soil). Results are expressed as means ± standard deviations. (Lukkari et al. 2004)

Phase II biotransformation involves mostly conjugations to more polar compounds. In particular, Glutathione S-Transferases can catalyse the conjugation of a wide range of alkylating compounds such as many xenobiotics, but also endogenous metabolic by-products to Glutathione (Boyland & Chasseau 1969). GSTs can be either in a cytosolic form or membrane-bound, the latter usually accounting for 10% of the total activity (Jakobsson et al. 1999). Its overproduction is involved in the resistance of pests populations to insecticides (Bass & Field 2011; Mamidala et al. 2012; Carvalho et al. 2013). Activation of GST in earthworms was demonstrated following exposure to pesticides. Schreck et al (2008) showed that a short-term (3 days) exposure of *A. caliginosa* to the insecticides chlorpyrifos and lambda-cyhalothrin increased GST activity. This indicated that GST participated in the detoxification of the selected insecticides. Long exposure or high dose of pesticides can also overcome the defences, and the enzyme itself can be affected. This was the case for a high dose or longer (14 days) exposure in the study by Schreck et al (2008), who observed a subsequent decrease of GST activity. GSTs have never been used to study acclimation or adaptation in earthworms and their broad response to pesticides makes them be a valuable parameter to assess acclimation or adaptation strategies.

As stated before, insecticides can have drastic impacts on earthworms. In particular, most organophosphates, carbamates or pyrethroid insecticides target Acetylcholinesterase, which is a vital enzyme degrading the neurotransmitter acetylcholine (Denoyelle et al. 2007). These pesticides hence act through neurotoxicity. Carboxylesterases are another group of serine hydrolases involved in the specific detoxification of such insecticide compounds. They hydrolyse efficiently synthetic pyrethroids and carbamates and further bind irreversibly to organophosphates (Sanchez-Hernandez et al. 2009). In aphids, modified carboxylesterases have been shown to be responsible for genetically-mediated resistance to organophosphate, carbamate and pyrethroid resistance (Devonshire 1977; Devonshire & Moores 1982).
Carboxylesterases have been described in a few study in earthworms (Sanchez-Hernandez & Wheelock 2009; Sanchez-Hernandez et al. 2009; Collange et al. 2010; González Vejares et al. 2010), and they would prove interesting indicators in studying acclimation or adaptations to insecticide resistance, for example in earthworm populations from insecticide-impacted orchards.

Heat-shock proteins (HSP) are families of proteins involved in folding and refolding of proteins either in statu nascendi (recently synthesized) or when damaged. Their induction by means of "stress genes" (Gupta et al. 2010), by a wide variety of stress factors, such as temperature and exposure to xenobiotics, salt or irradiation, have lead ecotoxicologists to use them as non-specific, general stress biomarkers (Köhler et al. 1999). Köhler *et al* (1999) showed that collembolas from a long-term metal contaminated site had elevated levels of HSP70 compared to reference (least contaminated site) populations.

Many biochemical pathways in aerobic organisms involve the production of Reactive Oxygen Species (ROS). These highly reactive compounds are usually counteracted by cellular mechanisms i.e. "antioxidants" when the balance between antioxidants and "prooxidants" (ROS) is favourable. However environmental stressors such as pollutants can act as pro-oxidants and shift this balance, thus inducing accumulation of ROS which is called oxidative stress. Wiegand *et al* (2007) demonstrated that exposure to paraquat, an oxidative stress-inducing herbicide, induced antioxidant defences in the aquatic oligochaete worm *Lumbriculus variegatus.* Oxidative stress can then result in physiological damages to cell components such as lipid peroxidation or DNA damage. One of the major antioxidant

enzymes is Catalase (CAT) that catalyses the dismutation of H202 (hydrogen peroxide, which belongs to the ROS) into O2 and H20, thus mitigating oxidative stress. In the nematod *Caenorhabilis elegans*, Sampayo *et al* (2003) showed that synthetic mimetics of CAT conferred enhanced resistance to the oxidative stress-inducing pesticide paraquat. Thus the investigation of oxidative stress-mitigating enzymes can provide insights into adaptation strategies to pesticides.

4.3 Quantifying energy reserves in contaminant-tolerant invertebrates to assess energetic trade-offs and "costs of tolerance"

Mechanisms of resistance vary with species and with the contaminants involved. However, they often imply overproduction of detoxification enzymes, which is postulated to have a "metabolic cost" to the animal in terms of energetic resources (Calow 1991). Organisms like earthworms are constantly in contact with the soil and ingest large amounts of it. Moreover, they usually move as little as 9m a year for *L. terrestris* (Lavelle & Spain 2001) which suggests that metabolic protection mechanisms would be the main strategy involved in possible adaptation processes, as opposed to behavioural (avoidance) ones.

Energetic reserves have been the subject of several studies on a common bioindicator of polluted estuaries, the ragworm *Nereis diversicolor*. Ragworm populations living in an heavily metal-polluted estuary (Restronguet Creek, UK) had acquired tolerance to cadmium, copper and zinc (Grant et al. 1989). On these populations, Pook et coworkers (2009) demonstrated significant costs, in terms of both energetics and reproductive outputs, associated with resistance to metal toxicity. The resistant animals had a significant lower scope for growth, associate with lower lipid and sugar body reserves, which led to a lower energetic content in oocytes.

These parameters indicated a higher allocation of energetic resources towards maintenance and metabolism, and less towards reproduction and anabolism.

Holmstrup et al (2011) demonstrated significant energetic costs in terms of glycogen use in the earthworm *Dendrobaena octaedra* associated to detoxification of metals. Glycogen levels were negatively correlated with Aluminium, nickel and zinc body burdens.

A growing field of ecotoxicology is the application of metabolomics, also called "ecometabolomics". Metabolomics aims at measuring responses of multiple variables from any tissue sample, which are mostly small molecular weight metabolites. These metabolites have great sensitivity in detecting phenotypic mechanisms and key molecules explaining organism's responses to environmental changes (Sardans et al. 2011). In the last five years, several studies have applied metabolomics to investigate earthworm responses to various pollutants such as heavy metals, pesticides, PAH, PCBs, and nanoparticles (Bundy et al. 2004; Jones et al. 2008; Yuk et al. 2011; Åslund et al. 2012; Whitfield Åslund et al. 2012; Mudiam et al. 2013). Metabolomics typically use NMR spectroscopy or Mass-Spectrometry (MS) based techniques (Lankadurai et al. 2013).

One of the main advances of metabolomics is that it can draw metabolic profiles and potentially discriminate between modes of actions. Guo et al (2009) indeed identified general stress responses and specific responses to toxicants with very different modes of action, i.e. the toxic metal salt cadmium, the pesticide atrazine and the polycylic aromatic hydrocarbon fluoranthene. An ongoing challenge is also to relate metabolomic changes to ecologically-related parameters and functional endpoints such as reproduction and weight change, which have direct relevance to population-level impacts. The already mentioned study by Guo *et al* (2009) successfully related metabolic responses (for the three toxicants) of earthworm *Lumbricus rubellus* to cocoon production, indicating general metabolic changes representative of overall stress and leading to a decrease in cocoon production. In the same species, Bundy *et al* (2008) coupled metabolomics to transcriptomics and functional ecological endpoints (reproduction rate and weight change) and demonstrated a disruption of energy metabolism following exposure to copper, which led to a reduction in reproductive parameters.

Bundy et al (2004) compared metabolic changes in indigenous (*Lumbricus rubellus* and *Lumbricus terrestris)* and introduced (*Eisenia Andrei*) earthworms along an environmental gradient of metal contamination. The native worms (*L. rubellus)* had elevated concentrations of maltose along the gradient of metal contamination, whereas introduced earthworms did not show such differences. They conclude that a potential biochemical response associated with metal tolerance occurs in earthworms from the highly contaminated site. This first study shows that metabolomics have great potential to be applied in studying adaptation to pesticides in earthworms.

4.4 Indicators and consequences at higher levels: individual and population

Exposure to pollution can affect higher levels of biological organization in organisms such as reproduction, growth and life history traits in general. In turn, organisms can be subject to selection by pollutant pressure on such traits. Several studies on metal adaptation of soil organisms have shown that resistance to metals can indeed be associated to changes in life-history traits that are beneficial in metal contaminated areas. Donker *et al* (1993) demonstrated that a metal-adapted population of the isopod *Porcellio scaber* had been selected for early reproduction and reproductive allocation, although there wasn't much difference in growth. However, this resulted in fewer young per females. Bengtsson et al (1992) compared populations of the worm *Dendrobaena octaedra* which originated from either a very long-term contaminated (at least two centuries), a long-term contaminated (20 years) and a non-contaminated area, by heavy metals. They showed that pre-adapted specimens reached a larger body size with a higher reproductive output than naïve ones when reared in the soil with the highest contamination levels. Fisker et al (2011) showed that populations of the earthworm *Dendrobaena octaedra* from a long-term polluted site exhibit a genetic resistance to copper. This resistance was studied by comparing pre-exposed (i.e adapted) versus "naïve" (unexposed) earthworms in polluted and control soil, and the authors showed higher individual growth, reduced time to maturity and increased reproduction in adapted compared to reference earthworms (Fisker et al. 2011). The adaptation was linked to elevated levels of the metal-mitigating enzymes metallothioneins (Fisker et al. 2012).

A considerable literature is available on pesticides impacts on earthworm growth and reproduction, recently reviewed by Yasmin and D'souza (2010). Numerous studies have reported loss of weight following exposure to herbicides, fungicides and insecticides, most of them on *Eisenia fetida*/*Andrei* (Haque & Ebing 1983; Bustos-Obregon & Goicochea 2002; Espinoza-Navarro & Bustos-Obregón 2005; Yasmin & D'Souza 2010). Along with weight changes, impairments in reproduction processes have an obvious interest in predicting changes at the population level. Indeed, even though population densities may not be immediately affected by a chemical exposure, adverse reproductive consequences may in time result in a reduction. Reproduction impairment can be assessed by measuring cocoon and hatchling production, the viability of the worms produced, sexual maturation, sperm count and sperm quality.

However, life history traits have never been used in an adaptation of earthworms regarding pesticides. Thus, such parameters could be very useful endpoints to investigate earthworm adaptation to pesticides.

5 Aim of the study and research work

In the process of homologation of new pesticides, in theory the most toxic compounds for earthworms are screened out by the available tests on *Eisenia fetida* based on mortality and reproduction. However as stated before, these tests may not take into account the more subtle effects of homologated pesticides:

i) on populations of more sensitive earthworm species
ii) as long-term contamination (i.e. several decades)

Despite the contamination, in soils that sustain heavy agricultural activity and repeated pesticide input, earthworm populations of a few species persist. This fact questions which strategies these populations have developed to maintain themselves in soils contaminated by pesticides and their residues, and to which extent acclimatisation and/or adaptation processes are involved. Furthermore, the interrogation raises to which costs, as tradeoffs, those strategies take place, for earthworm populations (energy allocation and life traits tradeoffs) but also for the soil ecosystem (bioturbation efficiency, C and N cycles dynamics, and pesticide degradation as an ecosystem service).

The aim of the present thesis is to assess such adaptation potential in earthworm populations by comparing populations from contrasted agricultural backgrounds in terms of pesticide applications. In particular the use of “reference” (form uncontaminated areas i.e. “naïve” animals) versus pre-exposed populations (from agricultural fields under conventional management) will provide insights in the earthworms’ adaptation potential to pesticides. This work attempts to investigate adaptive strategies at several biological levels of investigation, from the sub-organismal (biochemical, enzymatic, metabolic) to the individual (weight changes) and population (reproductive parameters) levels, as well as possible ecosystem consequences (earthworm burrowing and consequences for pesticide fate).

The first step will be to assess to which extent the agricultural history can be linked to pesticide contamination and residues in the soil. A comprehensive analysis of pesticide

residues will be realised and pesticides profiles will be compared between the different fields in order to select the fields most appropriate for the study.

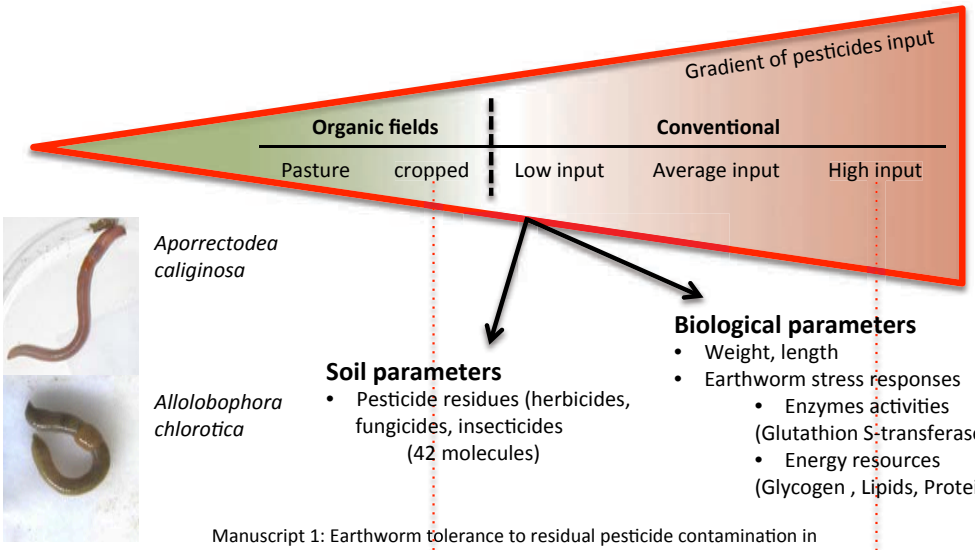
A detoxification enzyme with broad substrate specificity (GST, both as cytosolic and microsomal form) and a major anti-oxidant enzyme (CAT) will be used and compared within populations to assess if these “protection” mechanisms have improved in populations pre-exposed to pesticides compared to reference populations. Both these parameters will be tested as basal activities in field-sampled earthworms (i.e. constitutive expression without exposure) as well as induced following exposure to environmentally relevant concentrations (according to field application rates) of pesticides in laboratory experiments.

Energetic trade-offs of these protection mechanisms will be assessed via the status of main energetic reservoirs, which are glycogen (the main glucidic storage compound in animals), lipids, and proteins. The overall metabolic responses of the organisms will be evaluated by quantifying respiration rate, and by the use of metabolomics. The applied methodology allows the different measurements (respiration, energy resources, metabolomics) to be conducted on the same animal with several aliquots of lyophilized and ground powder.

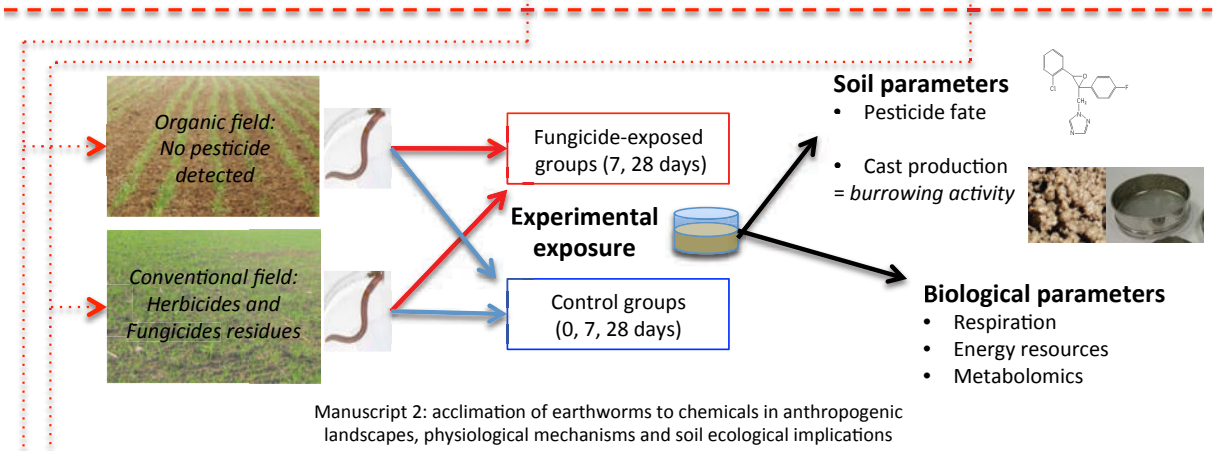
Consequences at a higher level of biological organization i.e. population level will be investigated through life-history traits. Several morphological and biometric traits will be compared in adults from the different agricultural fields as well as in their offspring (cocoons and juveniles).

The consequences of earthworm adaptation for the soil ecosystem will be addressed in terms of bioturbation (burrowing activity) and pesticide degradation. Burrowing activity of pre-exposed versus naïve earthworms will be quantified in laboratory experiments to attempt the link between adaptationto responses (e.g. metabolomics, energy resources, respiration rate) and soil bioturbation as a functional endpoint. Pesticide degradation will be compared in soils with pre-exposed and naïve earthworms, as well as in soil without animals (natural dissipation) to assess if earthworm adaptation can influence pesticide degradation as an ecosystem service.

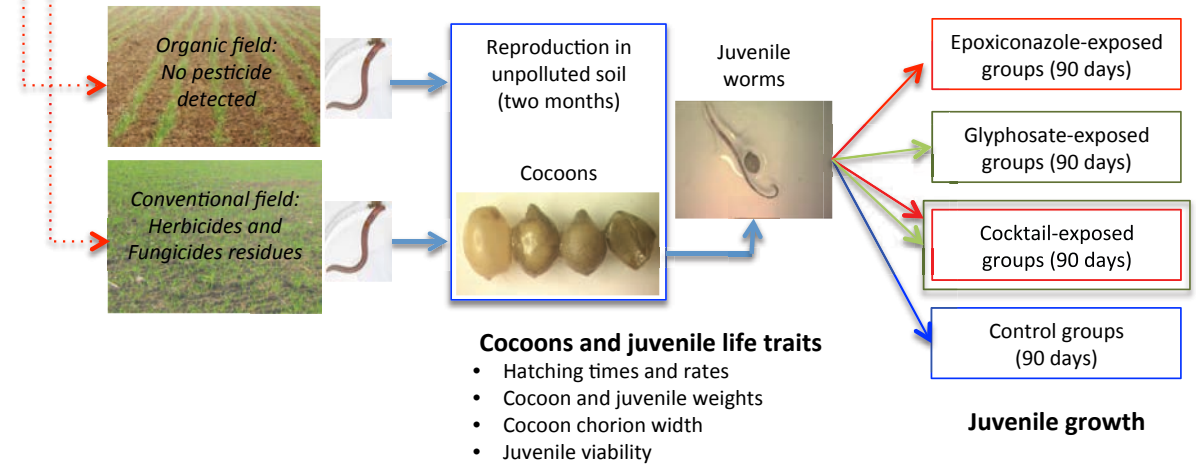
Graphical abstract



Manuscript 1: Earthworm tolerance to residual pesticide contamination in agricultural landscapes: from field assessment to physiological responses



Manuscript 2: acclimation of earthworms to chemicals in anthropogenic landscapes, physiological mechanisms and soil ecological implications



Manuscript 3: Life traits of cocoons and response to organic soil contamination in earthworm populations facing different agricultural practices

6 Agricultural context, earthworm populations, and model molecules overview

6.1 Study site and pesticide use

The selected agricultural fields are all located in the same agricultural basin (Vézin-le-Coquet, Brittany, France). They have slightly acid silt-clay-loam soils with no major difference in composition (see article 2). Three of the fields are conventional cropped (mainly cereals-maize-protein crops rotations) for more than 20 years with 5-6 pesticide applications per year, in addition to 1-2 times organic (pig manure) or chemical fertilization per year, and mechanical treatment using both mechanical and chemical weeding. They were selected according to their history of cultivation and pesticide applications since 2000, on the basis of similar crops and along an increasing gradient of the total sum of pesticide input (in g/Ha) of all active molecules applied (2000-2010) and thus designated as 1. "high-" (>18.2 kg Σ(active ingredients)), 2. "medium-" (>7.5 kg Σ(active ingredients)) and 3. "low-input" (>3.2 kg Σ(active ingredients)) fields. Although the total sum of active ingredients was very different between the fields, they did not differ much in terms of mean number of applications as shown in table 4:

Table 4 : mean number of pesticide applications (2001-2010)

on the fields under conventional management.

Pesticides applied	**Mean number of applications per year**		
	Low-input	**Medium-input**	**High-input**
herbicides	**3.4**	**3.2**	**2.3**
fungicides	**1.0**	**1.9**	**1.1**
insecticides/molluscicides	**0.3**	**0.6**	**1.2**
growth regulators	**0.2**	**0.1**	**0.2**
All types of pesticides	**5.0**	**5.8**	**4.9**

The first reference population (cropped but without pesticides) was a field cropped according to organic agriculture requirements since 1992 (mainly cereal-proteaginous rotations), where no chemical pesticides and only organic fertilizers were applied, but where mechanical weeding was performed.

The second reference population (uncropped, without pesticides) was a permanent organic pasture whether neither applications of pesticides nor soil tillage had been performed since 1960.

On the 57 different active molecules that had been applied on either of the three conventional fields between 2000 and 2010, were sorted according to criterias of frequency, amount, and last date of application on the three conventional fields 4 in an “index of relevancy” ranging from 0 to 4. The highest scores of the index are presented in Table 5. This index was used to determine which molecules to include in the soil screening (pesticide residual contamination) and to choose as “model” pollutants for the laboratory exposures. Hence we chose the model pollutants epoxiconazole and glyphosate (in bold, table 5) for the laboratory exposures for their high scores and because they had been the most frequently applied on the “high-input” conventional field, and are still widely used in france and worldwide.

Table 5 : top values of the "index of relevancy" of pesticides molecules applied on either if the three conventional fields.

Active molecule	**type**	**Index**
glyphosate	Herbicide	4
tebuconazole	Fungicide	4
Alachlore	Herbicide	3
cyprodinyl	Fungicide	3
Epoxiconazole	Fungicide	3
imazamétabenz	Fungicide	3
mercaptodimethur	Molluscicide	3
paraquat	Herbicide	3
pirimicarbe	Fungicide	3
sulcotrione	Herbicide	3

6.2 Overview of the two model agricultural pollutants epoxiconazole and glyphosate

Epoxiconazole (CAS n° 133855-98-8) is a large-spectrum systemic fungicide which belongs to the triazole family. It is used worldwide to control the development of parasitic fungi on many types of crops, and in France mainly on cereals, but also beetroot or corn (e-phy.agriculture.gouv.fr). The conazole fungicides in general prevent fungal growth by inhibiting the enzyme lanosterol 14-α-demethylase (CYP51) which controls the synthesis of ergosterol, an essential component of the fungal cell membrane (Kjærstad et al. 2010). It also acts on plants by causing several morphological and physiological changes allowing a better resistance against environmental stressors such as drought, high temperatures, and herbicide treatments (Wu & von Tiedemann 2001; Percival & Noviss 2008).

Epoxiconazole is suspected to be an endocrine disruptor. Conazole fungicides are known to influence the activity of cytochrom p450 enzymes, and in particular disturbing the activity of aromatase (CYP19) (Kjærstad et al. 2010). In rats, a perturbation of reproductive development was demonstrated: virilisation of female offspring, feminization of male offspring, fetotoxic effects at high dose and increased birth weights at low dose (Taxvig et al. 2007). Akcha et al (Akcha et al. 2008) evidenced that epoxiconazole had genotoxic effects on dinoflagellate *Karenia mikimotoi*, however genotoxicity evidence are still lacking on animals (AGRITOX 2011). Detoxification via cytochrome p450 followed by GST has been evidenced in rats, hence might occur in other organisms too (Hester et al., 2012). It is considered as "very stable" in soils (AGRITOX 2011) and has a reported field DT50 > 400 days (Kegley et al. 2011).

Glyphosate is a non-selective, systemic post-emergence herbicide used worldwide on many types of plants. Since its commercial introduction in 1970 it has become the dominant herbicide worldwide and accounts for 25% of the world's herbicide market (Duke & Powles 2008; glyphosate.eu 2013). Its metabolite AMPA was one of the main molecules found in rivers and estuaries of Côtes d'Armor, Bretagne, France (Conseil Général des Côtes d'Armor 2013).

It acts through inhibition of the enzymatic conversion of shikimic acid into anthranilic acid in the shikimate pathway, leading to accumulation of shikimic acid in the plant tissues (Steinrücken & Amrhein 1980). The shikimate pathway is unique for plants, fungi and bacteria for producing aromatic amino acids that are essential for animals. Hence, it is expected to have little impact on animals. However, adverse effects of glyphosate, particularly as commercial formulations, i.e with surfactants and other compounds added to the active ingredients have been evidenced in animals (Glusczak et al. 2006; Contardo-Jara et al. 2009; Modesto & Martinez 2010). Glyphosate and its formulation Roundup Ultra elevated levels of glutathione S-transferase enzyme in the black worm *Lumbriculus variegatus*, noteworthy, the formulation always causing higher enzyme activities (Contardo-Jara et al., 2009). One study on the earthworm *Eisenia fetida andrei* (Casabé et al. 2007) showed that a formulation of Glyphosate applied at recommended rate alters membrane stability as shown by the Neutral Red Retention Time (NRRT) assay.

Epoxiconazole was applied 4 times (2004, 2006, 2008 and 2010) on the "high-input" conventional field at the recommended application rate of 125g/ha. It was applied as commercial formulas Opus® and Ogam®. Glyphosate was applied three times (2004, 2006 and 2009) at different application rates depending on the culture (4 kg/ha, 2 kg/ha, and 2kg kg/ha) as Roundup 680®, RoundUp Flash® and RoundUp Max®.

For the laboratory exposures we used the field application rate of 125g/ha (1l/ha) for epoxiconazole (OPUS®), and 1.8 kg/ha (4l/ha) for Glyphosate (RoundUp Flash®).

Discussion and perspectives

I. Agricultural backgrounds and pesticide profiles

There is currently a lack of available information on soil contamination by pesticides. Pesticides, although they are applied to crops, are not often measured in the soil, but rather in plants and groundwater (Van-Camp et al., 2004). No detailed measurements of pesticide in soils are available at EU scale to evaluate the residual contamination by pesticides. One way to estimate pesticide residues in soils is via modelling (European Commission, 2004). A simple model of herbicide contamination in soils of European countries has been performed in 2004 by the Land Resource Management Unit of the European Commission (2004). The model predicted that under current agricultural practices several countries of the EU would exhibit increasing trends of herbicide quantities in soils under cereal, maize and sugarbeet cultivation, including France. From the history of cultivation of the three conventional fields, more than 50 different active molecules of pesticides had been applied at different application rates and frequencies between 2000 and 2010. However only 9 of them were detected or quantified in the soil in 2011, indicating large differences in the molecules' fates in the soil (manuscript 1).

From the pesticide molecules that were applied recently (2009-2010) on the fields, 2 out of 6, 3 out of 4, and 1 out of 3 were recovered using a "light" water extraction (i.e. representing the bioavailable fraction) in the "high-", "medium-" and "low-" input fields, respectively. In particular, dicamba, mesotrione, nicosulfuron, sulcotrione, cyprodinil, and bentazone were never found indicating that their persistence in soil is low or that they form unextractable residues. More concern should arise on the molecules in actual use that were the most frequently detected, alachlore and epoxiconazole. Epoxiconazole was detected in the three conventional fields, as it was applied recently. Alachlore was also detected in the three fields, although it hadn't been applied after 2000 in the "high-" and "medium-" input fields. Our study hence supports the high persistence of alachlore in the particular context of silty-clay loam soils. In the low input field, its concentration was still 0.3% of the amount of the last application in 2007, before it was banned for agricultural use in France and the European Union in 2008.

Atrazine persistence is now a well-known fact and its use is prohibited since 2003. In a recent study, Jablonowski *et al* (2008) demonstrated that up to 25% of ^{14}C-labeled atrazine was still present 22 years after the last application on a gleyic cambisol agricultural soil of Germany. Atrazine is still a major pollutant of rivers and ground waters in France: in 2011, ten rivers of Brittany contained the metabolites 2-hydroxy atrazine and atrazine desethyl as the second and third most frequently detected molecules (more than 50% of the water samples), just after the glyphosate metabolite AMPA. The parent compound was only found in 11% of the water samples (CORPEP 2011).

It should be noted that the analytical procedure used in this study (manuscript 1) involved a "light" extraction technique (water extraction) designed to yield the easily extractable, thus bioavailable fraction. As shown by Gevao *et al* (2001), uptake by earthworms through epidermis or soil ingestion (hence bioavailability) decreases with "aging" of the molecules in the soil, and their binding to soil particles. It is possible that several molecules were not recovered by water extraction because they form bound residues in the soil. For instance, dicamba was last applied on the "high-input" conventional field in 2007 and was not recovered. It was classified by Mordaunt *et al* (2005) as undergoing fast degradation, but also forming a high proportion of bound residues (55-65% in 91 days) (See Introduction, 3.2.2). This study supports the assumption that contamination of surface waters by original compounds or metabolites of pesticides can occur through slow release of the compounds from bound residues in agricultural soils. Moreover, the exposure risk for soil organisms is probably higher than what can be expected from the available fraction since only nine molecules were recovered by the soil extraction.

II. Enhancement of detoxication capabilities in earthworms from conventional fields.

Significant differences in the activities of the detoxication and anti-oxidant systems Glutathione S Transferase and Catalase were observed between earthworm populations of the species *A. caliginosa* and *A. chlorotica* from contrasted agricultural backgrounds (manuscript 1).

In field-sampled individuals, activities of sGST and Catalase were higher with increasing historical inputs of pesticides in *A. caliginosa*, whereas in *A. chlorotica* mGST activity followed this trend. Earthworm sampling had taken place 8 months after the last pesticide applications on conventional fields (the last fungicide applications on the three fields had taken place in may-june 2010, and no herbicide was applied during autumn) and thus the enzyme activities were considered "basal" i.e. at a constitutive level. Constitutive increase in detoxication enzymes is a common mechanism of resistance to pesticides in pest insect populations. As early as 1977, Devonshire (1977) showed that the enzyme carboxylesterase was involved in the resistance to insecticides of the peach-potato aphid (*Myzus persicae*). It was later evidenced that the enzyme was present in all populations but over-produced in the resistant animals, possibly because of gene duplication (Devonshire and Moores, 1982).

In non-target populations "local adaptations" mediated by enhanced detoxication were demonstrated, especially for metals which are known to exert a significant toxic selection pressure (Pauwels et al., 2013). A metal-resistant population of the marine worm *Limnodrilus hoffmeisteri* was found to have genetically-determined elevated levels of the metal-binding proteins metallothioneins (Klerks and Bartholomew, 1991). However a difference in metal-related parameters observed in field-collected adults is not always transferred to the offspring, i.e. genetic adaptation is not demonstrated. Additional experiments with F1 or F2 generation, or investigations at the genetic level should be conducted to confirm the heritability of such changes. Rozen (2006) showed that adult earthworms *Dendrobaena octaedra* collected from a long-term cadmium-contaminated site exhibited higher cadmium storage capacity. However this pattern was not conserved in the offspring (F1 generation), showing that this accumulation capability of cadmium was acclimatory and not genetically adaptive. Fisker *et al* (2013) have very recently showed upregulations of metallothioneins genes in F2 generation

earthworms *Dendrobaena octaedra* from copper-resistant populations, demonstrating a clear genetic basis for metal resistance. This inheritability may be species specific and exposure scenario dependent. Activation of the GST in the pre-exposed population (from the "high-input" field) was then confirmed in an experimental exposure of *A. caliginosa* to a fungicide. This activation did not take place in the earthworms from the field under organic management (i.e. "naïve" animals) (manuscript 1). Hester et al (2012) demonstrated in rats that epoxiconazole triggered gene induction of several sGST isoenzymes, as well as a group of genes associated to oxidative stress. GSTs exist in a widespread range of organisms, and play a fundamental role in protection against endogenous or exogenous toxic chemicals (Sheehan et al., 2001). The present study shows that pre-exposure to pesticides enhance the biotransformation, hence elimination capacity of earthworms in conventional fields.

This study is the first showing an acclimatory response in the detoxification system of long-term pesticide-exposed earthworms. This acclimatory response could not only be based on GST (phase II detoxification mechanism), but possibly also on other phases of the biotransformation/detoxication system, such as cytochrome p450-related enzymes (i.e. phase I detoxication). The marked increase in soluble proteins in the pre-exposed earthworms when exposed to fungicide indeed supports the assumption that other parts of the detoxication system could be involved. The measurement of additional detoxification enzymes such as cytochrome p4501A (CYP1A) monooxygenase (Lukkari et al., 2004) would provide more insight into this acclimatory mechanism. A genetic basis could be assumed and would be very valuable to assess too. Further exposure experiments to the fungicide epoxiconazole, and possibly other pesticides (to test if this response is specific to one pollutant or to a whole family) could be realised with F1 and F2 generations. Investigations in gene expressions of glutathione S transferases following exposure to the fungicide would also provide valuable perspectives for this work.

III. Energetic and metabolic rearrangements associated to physiological adaptation

Xenobiotics challenge biological systems, and their effects can be mitigated by numerous mechanisms of protection such as detoxification as previously discussed. A common assumption is that these mechanisms are costly in terms of metabolic resources and especially energy (Calow, 1991). To investigate such energetic and metabolic costs, metabolic responses and energy allocation of earthworm populations from the “high-input” and the organic field were compared following exposure to the fungicide epoxiconazole, which the worms from the conventional field had been pre-exposed to (manuscript 2). Metabolic rates (as measured by respiration) were significantly increased in both populations, indicating an energetic rearrangement in both populations. Interestingly, the consequences on the main energy resources differed between populations. The rapid available glycogen resource depleted in both groups, but earlier in the naïve earthworms (7 days) compared to pre-exposed ones (28 days). Soluble protein content was on the other hand increased after 28 days in the pre-exposed earthworms only, indicating over-production of biological compounds, possibly including detoxication enzymes as stated in II.

Protein production is one of the most costly mechanisms in animals, only osmoregulation is comparable in terms of energy consumption (Smith and Houlihan, 1995; Wieser and Krumschnabel, 2001). Due to the high energy demands, protein synthesis can induce metabolic feedback mechanisms and homeostatic controls. Metabolomics allow to view a major part of the biochemical phenotype and can thus provide insights into these feedback mechanisms (Lankadurai et al., 2013). In our study, a general increase in most metabolites was observed in the pre-exposed population facing the fungicide epoxiconazole, especially amino-acids (manuscript 2). These metabolic changes led to a separation of metabolic profiles between exposed and control earthworms after 28 days. In the naïve population however, a slight increase in amino acids was also observed, but not to such an extent as in the pre-exposed population. Rochfort et al (2008) reported elevated levels of lactate, formate, alanine, glucose and maltose in worms from a conventional field compared to worms from an organic field. The authors hence suggested that the increase in levels of these biological compounds

could be used as a biomarker of stress. Increase in amino-acids levels was also observed in *Lumbricus rubellus* exposed to pyrene (polycylic aromatic hydrocarbon) (Jones et al., 2008), and *Eisenia fetida* exposed to endosulfan (Yuk et al., 2011). The pool of amino-acids is at the centre of metabolic processes during stress responses, and abiotic stresses often result in an accumulation of several amino-acids (Krasensky and Jonak, 2012; Lankadurai et al., 2013). It is likely that the increase in amino-acids observed in both our populations is a metabolic rearrangement as a general stress response for coping with pollutants, with a response much stronger in the pre-exposed population. This is the first study demonstrating energetic costs and metabolic rearrangements associated with acclimation to pesticides in earthworm populations.

IV. Consequences of pesticide use on earthworm populations

The two populations of *A. caliginosa* originating from the conventional and the organic field differed in terms of adult and cocoon traits, and in terms of response to pesticide contamination of the soil (manuscript 3). The capacity to reproduce is essential for the sustainability of field populations under heavy predation as they are at the basis of the food web (Khalil et al., 1996). Environmental stress factors can influence growth and reproduction of earthworms. A challenge is to understand how these influences can relate to changes at the population level because of the complexity and multiplicity of factors involved (Klok et al., 1997). Reproduction as measured by cocoon production rate was shown to be a sensitive parameter to assess the toxicity of chemicals, which led to the development of the earthworm reproduction test (Organisation for Economic Co-operation and Development, 2004). However other endpoints involved in reproduction success can indirectly impact population growth rates as e.g. cocoon and juvenile viabilities can be reduced by soil contamination. Casabé *et al* (2007) demonstrated that Glyphosate applied as commercial formulation reduced cocoon viability in the epigeic *E. fetida*, hence the number of juveniles. Gupta *et al* (2006) also showed that cocoon hatching rates in the endogeic earthworm *Metaphire posthuma* were severely decreased by exposure to metals. However in the case of the present study, the endogeic adults *A. caliginosa* had been exposed in the fields they originate from, but they were reared to reproduce in clean soil and cocoons were hatched without contaminants either. Still, hatching rate was markedly lower in cocoons obtained from the pre-exposed adults (manuscript 3). Hence the lower hatching rate observed is necessarily due to indirect effects, or adaptation processes in the adults.

Several authors reported that pesticides and metals can hamper earthworm growth at juvenile stage, hence delaying maturation (Klok and de Roos, 1996; Klok et al., 1997; Spurgeon and Hopkin, 1996; Spurgeon et al., 1994). In the present study, adults from the conventional field had a lower mean weight than the adults from the organic field, and this was repeatedly observed for the three sampling campaigns (article 1, 2 and 3). This indicates that the agricultural management, and in particular the use of pesticides leads to reduced mean weights, either by reducing individual growth rates as a result of higher energy demand

(energy costs mentioned in III) or favouring smaller individuals. It is to be noted that tillage was less frequent in the organic field during the lucerne periods, hence this parameter can also contribute to the reduction in mean adult weight in the conventional field. Cocoon size has been shown to be correlated with adult size in several species of earthworms (Lavelle, 1981; Reinecke and Venter, 1987; Edwards and Bohlen, 1996; Jiménez and Thomas, 2001). In the present study the cocoons from the pre-exposed adults had a significantly lower mean weight, although the juvenile weight did not vary (manuscript 3). It confirms that the difference in cocoon weights is linked to the parent's weights.

Earthworm cocoons contain an albuminous fluid that serves as feeding resource for the developing embryos (Lavelle and Spain, 2001). As the cocoon chorion thickness was the same in both populations, it is likely that the cocoons from the naïve population contained more nutritive fluids than the ones from the pre-exposed population, which was left unused upon hatching. West *et al* (2003) suggested that populations of the epigeic earthworm L*umbricus rubellus* stressed by soils with low calcium content allocate less energetic resources to reproduction, thus having smaller cocoons. It is possible that adult worms from the organic field are capable of depositing higher amounts of albuminous fluid, hence more energetic resources in the cocoon. Pesticide stressed adults may not be able to allocate as much energy to the cocoon as worms from a non-polluted environment because they have to allocate energy for detoxification (Manuscript 2). The higher amount of resources available in the cocoons from the naïve population could then facilitate their better hatchability (shorter time and higher success).

A few population growth rate models of earthworms under toxicant stress have been developed and suggest that population growth rates during exposure to pollutants are mainly influenced by development time (time to reach maturity) (Widarto et al., 2004; Klok et al., 2006), failure to reach adulthood (Klok and de Roos, 1996), length of the adult life span (Klok et al., 1997), fecundity (Widarto et al., 2004) and by the average time between production of cocoons (Widarto et al., 2004; Bindesbøl et al., 2007). However cocoon hatching rates are not taken into account, hence this study shows that population growth rates models could be improved by addition of juvenile viability as input parameters. A population

growth rate model by Klok and de Roos (1996) of the earthworm *Lumbricus rubellus* exposed to increasing concentrations of copper is presented in Figure 16.

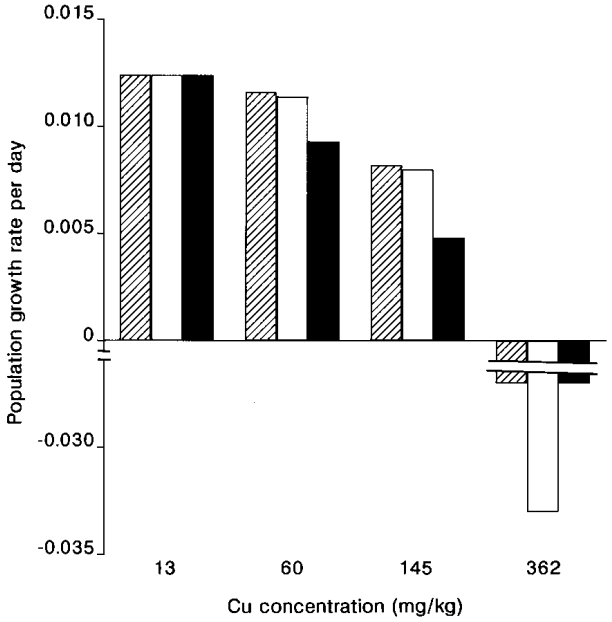


Figure 16: Effect of copper on the population growth rate per day of earthworm Lumbricus rubellus exposed to increasing concentrations of copper. Three possible scenarios of toxic influence are identified: (1) increase in maintenance requirements associated with detoxification (slashed bar), (2) decrease in the assimilation of energy (white bar), (3) increase in maintenance requirements for detoxification and an increase in the energy requirements to produce a single cocoon. Scenario 3 was the best fit to experimental results according to the authors (Klok and de Roos, 1996).

The present study adds to the knowledge that long-term exposure to pesticides in agricultural soils leads to a reduction in fecundity as measured by cocoon hatching rate, even when the adult worms are no longer exposed (i.e. cultured in clean soils). Two factors can then explain this reduction in fecundity. The observed lower fecundity is likely due either to reallocation of resources to metabolism associated to pesticide detoxification, or via an indirect effect of the agricultural management on adult weights, leading to lower cocoon reserves.

V. Consequences on soil functioning and ecosystemic services

There is an increasing need for a European reglementation in order to assess the risks of pesticides to human health and the environment (Reus et al., 2002). This calls for ecological risk assessments taking into account the different levels of biological organisation, with the challenge of relying sub-individual signals to ecologically relevant endpoints. Maboeta et al. (2001) showed that an application of the fungicide copper oxychloride induced a decrease in neutral red retention time (a biomarker of cellular damage) in South African grassland populations of the earthworm *Microchaetus sp*., followed by a decline of the population in terms of numbers and biomass. The earthworm numbers and biomass were still significantly lower after six months, even if copper concentrations in both soil and earthworms tissues had decreased significantly. Dittbrenner *et al* (2012) related an activation of stress proteins (HSP70) and at the same time an avoidance behaviour when exposed to the insecticide imidacloprid.

With regard to the ecological importance of earthworms through soil engineering and bioturbation, it is likely that, when attempting to assess the ecosystem services rendered by earthworms to the soil, earthworm burrowing behaviour is as important as population numbers as it can have drastic impacts for soil functioning (Lavelle and Spain, 2001; Capowiez and Bérard, 2006). The present study showed that activation of metabolic and enzymatic responses in earthworms and increase of their burrowing behaviour occurred simultaneously. Moreover, this pattern was only observed in the pre-exposed population, demonstrating that it is part of an acclimatory mechanism, possibly a compensating behaviour for higher energy demands. Measurements based on earthworm behaviour are still poorly used (Pelosi et al., 2013). Cast production is a new behavioural biomarker developed for toxicity testing (Capowiez et al., 2010). Burrowing behaviour of earthworms has been investigated by several authors in regard to pesticide contamination. The outcome of some of these studies was that pesticides such as organophosphates at or near field application rates can decrease burrowing activity or modifies burrow characteristics (e.g. decrease in burrow lengths, total area, rate of burrow reuse, maximal depth…) (Capowiez et al., 2003; Capowiez and Bérard, 2006; Capowiez et al., 2006; Dittbrenner et al., 2011). The direct consequences of the cast production test for soil functioning have mostly remained theoretical, except for a

study by Capowiez *et al (2006*) who showed that decreases in burrow length and depth were correlated with lower gas diffusion in the soil for *Allolobophora icterica.*

The fate of pesticides in soil can be affected by earthworms bioturbation via several mechanisms. It increases pesticides sorption on soil particles on the long-term, leading to the formations of non-extractable residues. Therefore it can increase the pesticide persistence, as it was previously shown for atrazine (Farenhorst et al., 2000; Binet et al., 2006). On the other hand, earthworms' activity was also reported to stimulate microorganisms activity, and enhance the activity of atrazine- or MCPA-specific bacterial degraders, accelerating its mineralisation (Monard et al., 2008, 2011; Liu et al., 2011). Schreck et al (2008) also suggested that *A. caliginosa* participate in the breakdown of four fungicides (folpet, fosetyl-Al, metalaxyl, myclobutanil) and two insecticides (Chlorpyrifos-Ethyl and λ-Cyhalothrin), as the pesticides concentrations decreased faster with the presence of earthworms. In our study, concentration of water extractable pesticides was lower in the microcosms containing earthworms from the conventional field, which showed higher bioturbation (manuscript 2). The increase in bioturbation observed in these earthworm's microcosms suggest that they play a part in the pesticide's disappearance either by enhancing sorption or by enhancing microbial mineralization of epoxiconazole.

Earthworms from the conventional field had a higher metabolism both in control and fungicide exposed groups (manuscript 1). A simple calculation allowed to estimate carbon dioxide dissipation from the soil to the atmosphere and compare it between the conventional and the organic fields. Therefore, to estimate earthworm biomass from both fields we used the data from Pelosi *et al* (2009) on earthworm biomass from a conventional and an organic field located close to Paris with comparable soils properties in terms of clay, silt and sand contents and climate. The conventional and the organic fields had a mean earthworm biomass of 32.1 and 37.6 g/m^2, respectively. The basal respiration rate of the pre-exposed and naïve populations were 95 and 40 μg CO^2 per g FW, respectively. For the purpose of the calculation, we assumed it constant for the whole earthworm community of the field. Therefore, the average carbon dioxide dissipation rate was 3.2 g and 1.5 g CO^2 / m^2 on the conventional and the organic field, respectively. This shows that a higher carbon dissipation

occurs through higher metabolims in pre-exposed earthworms which could have significant consequences for soil carbon balance, considering that earthworms are the largest soil biomass.

Our findings show that earthworms from the conventional field have enhanced biotransformation and anti-oxidant capabilities, allowing them to cope with contaminants. However these mechanisms are at the expense of metabolic and energetic costs, and are associated to a lower fecundity (as measured by cocoon hatching rate and time), which can lead to a decrease in earthworm densities in the fields. The population facing soil contamination exhibit higher bioturbation activity in microcosms, which in turn acts on pesticide dynamics. Little evidence is available in the literature on possible higher bioturbation activity following exposure to pesticides, so this pattern would need to be verified at the field scale before assessing consequences in terms of ecosystem services.

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Manuscripts

Manuscript 1:

EARTHWORM TOLERANCE TO RESIDUAL PESTICIDE CONTAMINATION IN AGRICULTURAL LANDSCAPES: FROM FIELD ASSESSMENT TO PHYSIOLOGICAL RESPONSES

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Earthworm tolerance to residual pesticide contamination in agricultural landscapes: from field assessment to physiological responses

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Abstract

This study investigates if long-term residual pesticide contamination in agricultural soils induces physiological changes in earthworm populations. Five fields were selected within a joint agricultural area (acidic loamy-clay soils) exhibiting different chemical and farming histories from conventional cultivation to organic pasture. Soil pesticide analysis revealed up to 9 molecules in low levels, including atrazine. Comparing pre-exposed population (conventional farming) to naïve (organic farming), exposure history of the endogeic *Aporrectodea caliginosa* and *Allolobophora chlorotica* modified their responses to pesticides. Activities of soluble glutathione-S-transferases (sGST) and catalase increased with soil pesticide contamination in *A. caliginosa*. Pesticide stress was reflected in the energy reserves of *A. chlorotica*. Upon laboratory exposure to an environmentally realistic concentration of the fungicide epoxiconazole, sGST activity increased in the pre-exposed population, but not in the naïve one. Pre-exposure to pesticides in the fields enhance the detoxication system of earthworms via the activation of sGST.

Keywords : *Aporrectodea caliginosa*, pesticides, biotransformation, oxidative stress, adaptation

Capsule

Exposure history of earthworms modifies their responses to pesticides in terms of activation of detoxication capacities

Highlights

- Analytical methods for 42 pesticide molecules and glyphosate/AMPA are presented
- Pesticides in fields affect physiological responses in earthworms species specific
- Pre-adapted population’s detoxification enzyme responds faster to pesticide stress

1. Introduction

In modern agriculture, cultivated fields are manipulated agroecosystems in which high yields are achieved through conventional management strategies based on the use of pesticides and fertilizers besides mechanical treatment. The repeated application of pesticides has led to chronic contaminations of cropped soils and thereby of their biota such as earthworms (Lumbricidae) (Lee, 1985; Redondo et al., 1994; Luchini et al., 2000; Gevao et al., 2001; Al Mughrabi and Qrunfleh, 2002). The persistence of several of them, such as the herbicide atrazine (Solomon et al., 1996; Giddings et al., 2005), raises environmental concern not only about residual contamination in cropped soils and their leaching potential to water bodies, but also about adverse effects on the soil biota.

Earthworms often represent the largest soil biota biomass and are commonly entitled "ecosystem engineers". They contribute to pedogenesis and influence many key parameters of the soil, such as nutrient availability and turnover, soil porosity, and microbial activity (Jones et al., 1997; Binet et al., 1998; Monard et al., 2008; Bottinelli et al., 2010). Earthworms' biodiversity however is reduced in intensively cultivated fields (Smith et al., 2008), with the use of pesticides evidenced as one of the responsible factors in laboratory toxicity studies (Springett and Gray, 1992; Yasmin and D'Souza, 2010). Consequently, a reduction in pesticide input could increase earthworm populations again (Pelosi et al. (2013a). Despite the hazards of these chemicals on earthworms, some species persist in intensively cultivated fields, in particular *Aporrectodea spp* (Jordan et al., 2004; Smith et al., 2008). This strongly suggests that long-term impacts of pesticide residues on earthworm communities are likely to induce adaptation processes, similarly to the described adaptations to metals (Posthuma and Van Straalen, 1993), including physiological and behavioural changes that may have consequences for the soil functioning, yet they are still poorly understood.

Earthworms' exposure to xenobiotics including pesticides can occur directly via uptake from soil interstitial water (porewater) through the outer skin and from ingestion of contaminated soil particles through gut epidermis (Belfroid et al., 1993, 1996). Exposure to pesticides then induces biotransformation and detoxication mechanisms in earthworms, like in other organisms, to alter or scavenge them, aiding excretion (Rodríguez-Castellanos and Sanchez-Hernandez, 2007). A constitutive increase in detoxication enzymes such as cytochrome P450

monooxygenases, esterases or glutathione-S-transferases can allow a multiple tolerance to contaminants in invertebrate populations, not only in target, but also in non-target organisms (Field and Foster, 2002; Penilla et al., 2006; Huang and Han, 2007; Brausch and Smith, 2009).

The metabolism involved in increased detoxication is costly in terms of energy (Medina et al., 2007; Fisker et al., 2011). As a result, reductions in annelids' energy resources have been associated with detoxication of pollutants: in *Dendrobaena octaedra*, internal regulation of metals like Al or Ni depleted glycogen (Holmstrup et al. (2011). In metal-resistant *Nereis diversicolor*, lipids, carbohydrates and consequently growth and fecundity were markedly reduced ((Pook et al., 2009; Holmstrup et al., 2011).

Aim of this study was to assess the capacity of earthworms to adapt and face residual soil contamination, which is a key issue to predict ecosystem sustainability and resilience. Energetic resources and antioxidant and detoxification enzymes of chronically exposed worms (from conventionally farmed fields) were compared to worms that had never been exposed (from biological farmed fields). In a first step the hypothesis was tested in field observations if long-term (>10 years) residual contamination of cropped soils manifests in physiological differences in earthworms. In a second step, it was validated in a laboratory exposure to environmentally relevant concentrations of pesticides on pre-exposed and naïve populations of *A. caliginosa*, if pre-exposure in the field impacts earthworms' physiological responses to the pesticides.

2. Material and methods

2.1. Field sampling (worms and soils)

Earthworm and soil sampling was conducted in five fields located in the same agricultural basin (Vézin-le-Coquet, Brittany, France) with slightly acid silt-clay loams (Table S1).

Three fields had been cropped according to conventional farming (with applications of pesticides and chemical fertilizers), one was cropped according to organic agriculture requirements (without any pesticide and using organic fertilizer), and one was an organic

pasture (uncropped). All fields and the pasture were in these types of management for at least 20 years.

The three conventionally cropped fields were selected according to their history of cultivation and pesticide applications since 2000, on the basis of similar crops and along an increasing gradient of the total sum of pesticide input (in g/Ha) of all active molecules applied (2000-2010) and thus designated as 1. “high-” (>18.2 kg Σ(active ingredients)), 2. “medium-” (>7.5 kg Σ(active ingredients)) and 3. “low-input” (>3.2 kg Σ(active ingredients)) fields. The conventional fields had been cropped with rotations of corn / cereals (Wheat, triticale, buckwheat, and barley) / protein crops (peas and horse beans) with the exception of flax in 2005 in the “medium input” field. The organic field had been under rotation of cereals and lucerne.

In spring of 2011, before the annual pesticide applications on conventional fields, soil and earthworms were sampled in five points disposed as quincunx separated by 25 m from each other. For pesticide and pedological characteristics analyses, 6 soil cores per sampling point were taken with an auger at 10 cm depth and pooled as a composite sample. Composite samples were passed through a 2mm sieve, dried for 24 hours at 30 °C and kept at -20°C until analysis. The two endogeic species *Aporrectodea caliginosa* and *Allolobophora chlorotica* were commonly found in all fields and the pasture, except *A. chlorotica* in the organic field.

In the same area where the soil cores had been taken, 7-9 earthworms from each of the two species *A. caliginosa* and *A. chlorotica* were sampled by hand-sorting. They were brought back to the laboratory in humid soil from their fields, rinsed and left for 24 hours for gut voiding, thereafter weighed, photographed (for length measurements using ImageJ software 1.440, (Rasband, 2012) and frozen in liquid nitrogen. A “condition” index of the animals was computed by dividing the weight of the animals by their length (Table 1).

Table 1: mean ± standard deviation (N=37-45) of weights, lengths and condition index of the two earthworms species sampled on the five fields in spring 2011. Different letters in italic denote significant statistical differences between the fields for each parameter.

Species	Parameters	Conventional			Organic	
		High-input	Medium-input	Low-input	Organic Field	Pasture
A. caliginosa	Weight	0.60 ± 0.12 *(bc)*	0.64 ± 0.11 *(b)*	0.79 ± 0.16 *(a)*	0.52 ± 0.16 *(d)*	0.55 ± 0.10 *(cd)*
	Length	8.08 ± 1.52 (a)	8.45 ± 1.59 *(a)*	8.63 ± 1.35 *(a)*	6.60 ± 1.33 *(b)*	7.77 ± 1.58 *(a)*
	Condition index	0.08 ± 0.01 (b)	0.08 ± 0.01 *(b)*	0.09 ± 0.02 *(a)*	0.08 ± 0.02 *(b)*	0.07 ± 0.02 *(b)*
A. chlorotica	Weight	0.27 ± 0.06 (b)	0.28 ± 0.05 *(b)*	0.33 ± 0.07 *(a)*		0.26 ± 0.05 *(b)*
	Length	4.43 ± 0.97 (a)	4.69 ± 0.91 *(a)*	4.90 ± 1.27 *(a)*		4.38 ± 1.07 *(a)*
	Condition index	0.06 ± 0.01 (ab)	0.06 ± 0.01 *(b)*	0.07 ± 0.02 *(a)*		0.06 ± 0.02 *(b)*

2.2. Pesticides extraction and soil residual contamination

Two analytical protocols were optimised, a multi-residue method targeting 42 pesticide molecules (19 of them had been applied on at least one of the three conventional fields), and a second specific protocol for the analysis of glyphosate and its metabolite AMPA. For the multi-residues, a liquid-liquid extraction was used, whereas glyphosate and AMPA were enriched via solid phase extraction. All pesticide molecules were analysed using LC-MS. Pesticide extraction, analytical procedures and conditions are detailed in the supplementary material. Analysed molecules, recovery rates, LOD, and LOQ are described in table S2. The two most quantitative and frequently applied active molecules in the history of the “high input” field were the fungicide epoxiconazole and the herbicide glyphosate. Hence these two molecules were chosen for the exposure.

2.3. Experimental setup of the earthwoms’ exposure

Soil used for the exposure was collected from the first 30cm of the organic pasture, gentle air-dried to 14% of humidity, then sieved at 2mm and kept in closed bins until start of the experiment. Adults and sub-adults (presence of reproductive characters) of *Aporrectodea*

caliginosa from the "high input" field and the organic field were sampled again in spring 2012 and acclimatized for 14 days to the exposure soil in the climatic room (Conviron GR96; temperature: 15°C; day/night cycle: 16/8h; humidity: 80 ± 5%) and individual weights were recorded (table S4). The two most frequent pesticides were applied (alone or in combination) as commercial formulations OPUS® (125 g active ingredient l^{-1}, BayerCropScience) and RoundUp Flash® (450 g active ingredient l^{-1}, Monsanto). Opus® and RoundUp® were applied at predicted soil concentrations of the active ingredient of 0.1 $\mu g.g^{-1}$ dry soil and 2.5 $\mu g.g^{-1}$ dry soil, calculated for field application rates of 1 $l.ha^{-1}$ and 4 $l.ha^{-1}$, respectively. Concentrations were calculated assuming a single application with an homogenous distribution, a soil density of 1.5 $kg.l^{-1}$, and no crop interception in the top 5 cm of the soil (Dittbrenner et al., 2010). Soil spiking was conducted by manually adding diluted pesticide solution or distilled water (for the controls) on soil at 14% water content reaching a final soil water content of 24%, in two steps with the soil being thoroughly mixed, redisposed as a fine layer and resieved at 2mm. Soil microcosms (polycarbonate boxes, 80mm diameter x 50mm height, Caubère, Yebles, France), were filled with 100 g of contaminated or control soil and left two days in a cool dark room to ensure aeration of the soil after re-humidification. Water content was adjusted to 25% prior to introduction of animals, then checked again each week.

Experimental design: Prior exposure, each earthworm was rinsed in tap water, gently dried on filter paper and put in individual Petri dishes for 48 hours for gut voiding. Then animals were individually placed in the exposure microcosm (day 0) according to a size-class procedure, insuring a similar mean earthworm weight in each treatment. Six earthworms were exposed to either soil with pesticide (herbicide, fungicide or the mixture) or control soil for 3, 7, and 28 days, with an initial control group for both populations (unexposed worms) at day 0. At end of each exposure time, earthworms were rinsed thoroughly, weighed again (table S4), frozen in liquid nitrogen and stored at – 80 °C.

2.4. Enzyme activities

Enzymes were extracted according to Wiegand et al (2007). Glutathione S-transferase activity in soluble and microsomal fractions (m- and sGST) was determined according to Habig et al. (1974), using 1-chloro-2,4-dinitro-benzene as substrate. Catalase (CAT) activity was measured according to Chang and Kao (1998), using H_2O_2 as substrate. Protein content of the

enzyme extracts was quantified according to Bradford using a calibration curve of bovine serum albumin (1976).

2.5. Energy resources measurement

The freeze-dried earthworms were grounded to a fine powder in a bead-beater (Retsch MM400, Retsch GbmH, Haan, Germany) and separated in aliquots: 2 mg for total lipids, 5 mg for soluble proteins, and 20 mg for glycogen measurements. Methods for lipids (Folch, 1957), proteins (Bradford, 1976) and glycogen (Nicolai et al., 2012) measurements are described in detail in the supplementary materials. Whole-body-energy-budget (WBEB) was calculated as sum of combustion of 17kJ/g for glycogen, 39.5 kJ/g for lipids, and 24 kJ/g for proteins (Smolders et al., 2003).

2.6. Statistical analyses

Box-and-whiskers plots represent the data quartiles and the median (black bar). The length of the box represents the Inter-Quartile Range (IQR), and whiskers extends to the data points that are no more than 1.5 times the IQR from either end of the box. Mean differences in enzymatic (sGST, mGST, and Catalase), energetic (Glycogen, lipids, proteins, and WBEB), and biometric parameters (length, weight, and condition index) between the fields were tested by one-way ANOVA with field residual contamination as factor, followed by Tukey's post-hoc test. Weight changes of earthworms in the laboratory exposure were tested by one-way ANOVA with time as factor, followed by Tukey's post-hoc test. At field scale, relationships between earthworm responses in terms of energy and enzymes activities and soil residual contamination were analyzed by PCA. Data was log-transformed, then Principal Component Analyses (PCA) were performed on the standardized *A. caliginosa* and *A. chlorotica* datasets from all the fields except the "average input" conventional field that was not finally retained in the analysis for better clarity.

3. Results

3.1. Pesticide residues in agricultural soil

Nine pesticides were detected in the soil of either of the five fields, 5 herbicides, 3 fungicides, one metabolite of glyphosate, but no insecticide (Table 2). 6, 8 and 4 residues were recovered in the "high-", "medium-", and "low-input" fields, respectively. From the molecules that were applied recently (2009-2010) on the fields, 2 out of 6, 3 out of 4, and 1 out of 3 were recovered. Several molecules were applied in 2009-2010 and, although they were included in the analytical method, were not recovered in any of the three soils, e.g. dicamba, mesotrione, nicosulfuron, sulcotrione, cyprodinil, and bentazone. Only the metabolite of glyphosate was detected in two fields (AMPA) but not the parent compound.

Table 2: mean and standard deviations (out of three replicates) of pesticide residues concentrations in the soil of the five fields studied in $ng.g^{-1}$ dry soil and number of detections (out of the 25 sampling points). In grey, pesticides that had not been applied since the start of the available cultivation history (2001). pesticide type: h=herbicide, f=fungicide, m=metabolite, LOD = limit of detection, nd = below detection limit, <LOQ =detected but below quantification limit

Active molecule	**Type**	**LOD**	**High input**	**Medium input**	**Low input**	**Organic field**	**Pasture**	**Number of detections**
Atrazine	h	0.4	< LOQ	1.6 ± 0.2	2.4 ± 0.5	< LOQ	nd	17
Alachlore	h	0.8	2.9 ± 0.6	4.2 ± 0.4	8.8 ± 3.1	nd	nd	15
Epoxiconazole	f	0.4	11.1 ± 2.7	4.4 ± 1.1	4.2 ± 0.5	nd	nd	15
Cyprodinyl	f	0.8	< LOQ	< LOQ	nd	nd	nd	10
AMPA	m	50	117.5	65.9 ± 8.7	nd	nd	nd	6
Chlortoluron	h	1.7	nd	6.4 ± 1.8	nd	nd	nd	5
Simazine	h	0.4	nd	< LOQ	nd	nd	nd	5
Tebuconazole	f	1.7	nd	< LOQ	< LOQ	nd	nd	5
Azoxystrobine	f	4.2	< LOQ	nd	nd	nd	< LOQ	2

The herbicide atrazine was present in the three conventional (from 1.33 to 2.84 $ng.g^{-1}$ dry soil) and even detected in the organic field (below LOQ), but not in the pasture. It was thus the most frequently detected, although it hadn't been applied during the ten years (2000 – 2010)

of available cultivation history of the three conventional fields. The two most frequently detected molecules of pesticides in actual use were alachlore and epoxiconazole. The epoxiconazole residues represented between 3 and 7 % of the predicted concentration calculated according to their field application rates (Dittbrenner et al., 2010) after the last application of the pesticide, (2010 in the high input, 2009 in the medium input and 2007 in the low input field). Alachlore was not applied after 2000 on the high or medium input fields. In the low input field, its concentration was still 0.3% of the amount of the last application in 2007, before it was banned for agriculture use in France in 2008. Chlortoluron was detected in the medium-input field only after its application one year before. AMPA was found in the medium input field, and scarcely found in other fields. However, applications of RoundUp Flash (glyphosate) had taken place on the three conventional fields in 2009 (medium and high input) and 2010 (low input). With the exception of the fungicide Azoxystrobine in one sampling point (<LOQ), no pesticides were detected on the pasture.

3.2. Enzyme activities in earthworms from the field

In *A. caliginosa* the activity of the detoxication enzyme sGST was constitutively increased at highest input of pesticides in the conventional fields, compared to low activities at the medium and low input fields, whereas the organic field and the pasture were intermediate (Fig 1). The mGST of *A. caliginosa* remained similar in the five fields. Almost opposite reactions were seen for *A. chlorotica*, where the mGST was constitutively increased with increasing pesticide content in the soil in conventional fields, and lowest in the pasture whereas the sGST didn’t follow a trend between the fields and the pasture. The catalase activity was constitutively more active in *A. caliginosa* with increasing pesticides in the soil, whereas it remained similar between the fields in *A. chlorotica*.

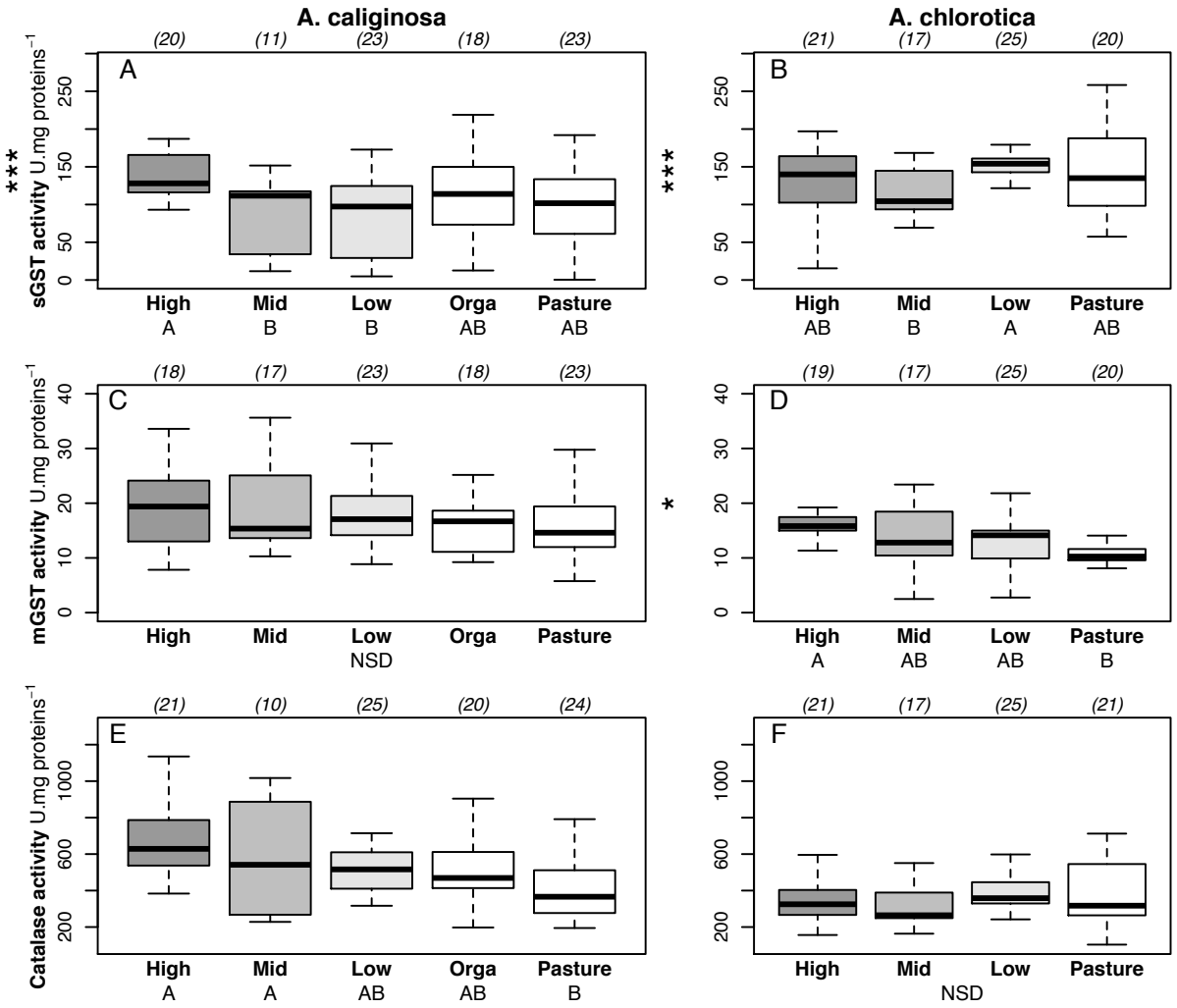

Figure 1: activities of soluble (sGST) and microsomal (mGST) Glutathione S transferase, and Catalase in earthworms *A. caliginosa* (left) and *A. chlorotica* (right). Significant impact of "soil contamination" is indicated by asterisks in each graph (* p<0.05, ** p<0.01, ***p <0.001). At the bottom of each graph, different letters (A, B or C) denote statistical differences between fields.

3.3. Energy resources in earthworms from the field

In *A. caliginosa g*lycogen content was lowest in the high-input field, and highest in the low-input field, but intermediary in worms from the organic field and the pasture (Fig 2A). Lipids and proteins showed the same pattern: highest in the medium-input one, and low in the organic, but medium to high in the pasture (Fig 2 C, E). Consequently the WBEB followed that trend (Fig 2 G).

In *A. chlorotica* glycogen, lipid and protein contents were lower in earthworms from the high-input field compared to the pasture. *A. chlorotica* from the medium- and low-input fields also had lower glycogen and protein content than the ones from the pasture. This was also reflected by the WBEB, which was significantly lower in the high-input and low input field compared to the pasture.

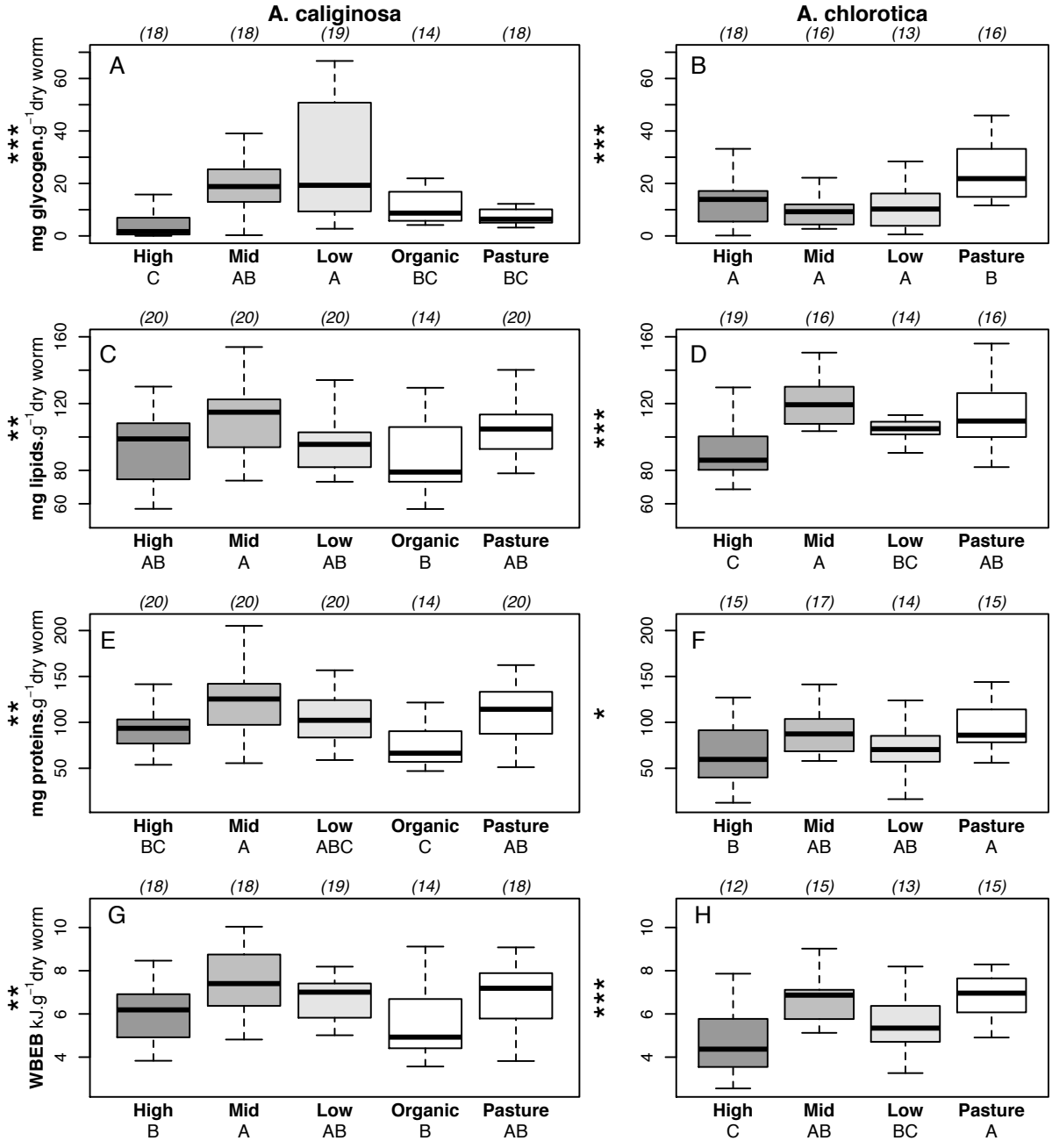

Figure 2: energy reserves (glycogen, lipids, proteins and whole-body energy budget) of earthworms *A. caliginosa* (left) and *A. chlorotica* (right) depending on field soil contamination (box plot with n values for each

in brackets). Significant impact of "soil contamination" is indicated by asterisks in each graph (* $p<0.05$, ** $p<0.01$, ***$p<0.001$). Different letters below field labels indicate significant differences as determined by Tukey's « Honest Significant Difference method ».

3.4. Earthworms' enzymatic responses after exposure to the two pesticides and their mixture

No differences in enzyme activities (sGST, mGST, CAT) were found between control groups of both earthworm populations at start of the experiment (Fig 3). Activities of the sGST differed greatly after experimental exposures between the two differently adapted populations. It increased after the fungicide treatment after 7 and 28 days of exposure in the pre-exposed population, but not in the naïve one (Fig 3 A, B). The herbicide did not provoke changes of the sGST activity in both populations. The mixture, however increased sGST activity after 28 days exposure in the pre-exposed group, compared to the naïve population, and compared to the activities after shorter exposure. The fungicide did merely not alter mGST activity, whereas the herbicide and the mixture decreased the activities of mGST, hence blocked this detoxification possibility (Fig 3 C, D). Catalase activity decreased significantly after three days exposure to the pesticides in the pre-exposed population, but only under epoxiconazole exposure in the naïve population (Fig 3 E, F). Activity of the catalase was elevated by the fungicide alongside an elevation in the controls in both populations for unknown reasons, hence not significant against the controls. The herbicide and the mixture again merely decreased the activity of this enzyme.

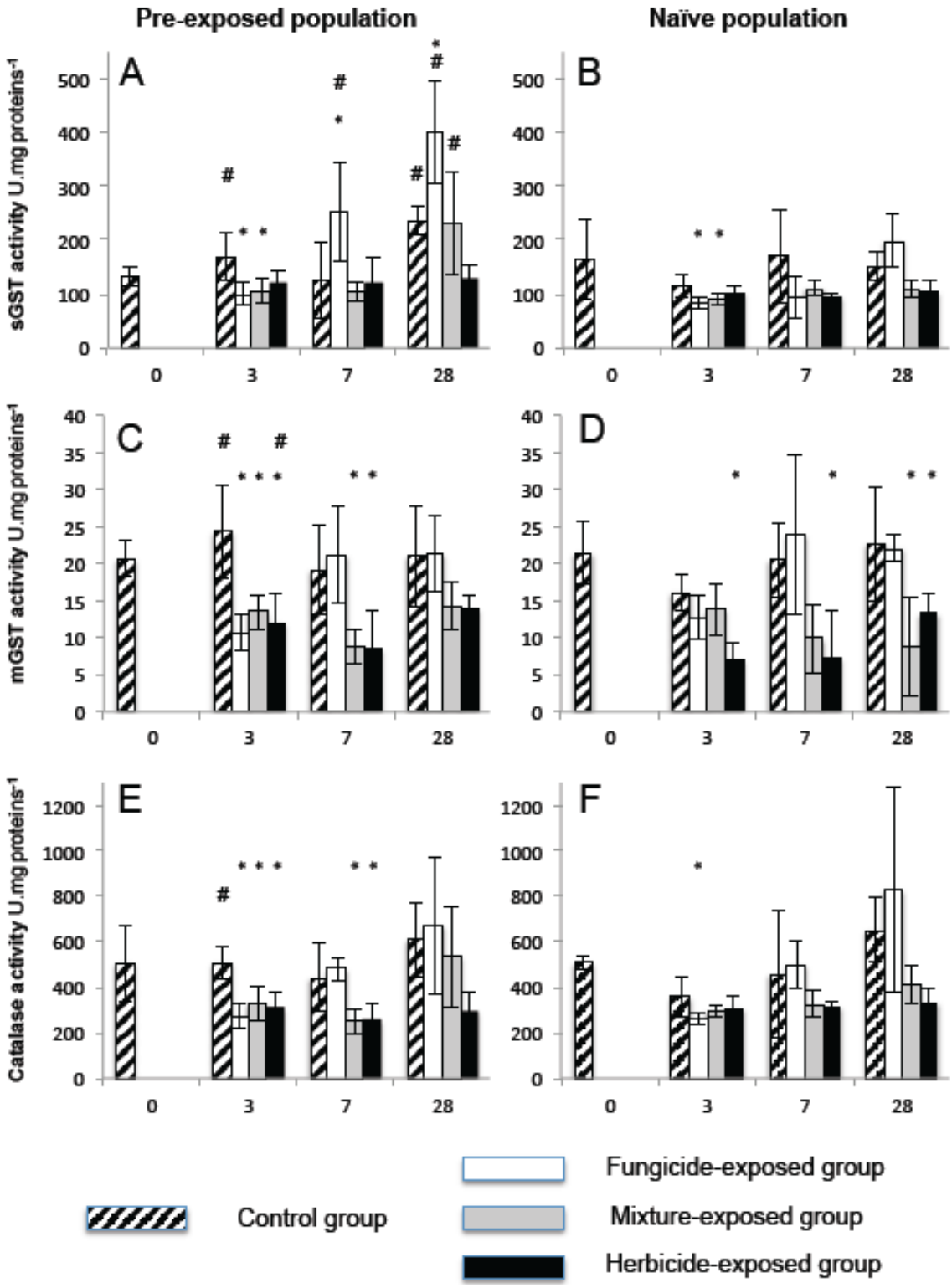

Figure 3: mean activities (± standard deviations as error bars) out of six replicates (N=6) of soluble and microsomal Glutathione S-transferase and Catalase in earthworms *A. caliginosa* after 3, 7 and 28 days of exposure to the fungicide Opus® (0.1 µg active ingredient epoxiconazole g^{-1} dry soil), RoundUp Flash® (2.5 µg active ingredient glyphosate g^{-1} dry soil), and their mixture (as added ingredients).

* indicate significant differences between the treated and the control group and # indicate significant differences between control groups of the naïve and pre-adapted population.

3.5. Earthworm physiology and field soil contamination

Scores plots of PCA on the *A. caliginosa* dataset separate the four fields from each other on axis 1 and 2 (Fig 4 A). The high-input field in particular is clearly separated from the pasture as it has high scores on Axis 2, whereas the pasture data points group in the lower half of the figure. The main drivers for this separation seem to be Catalase, soluble GST, proteins and lipids as shown in Fig 4 B by their correlation with Axis 2. The low-input field is characterised by negative scores on both Axis 1 and 2 (lower left quarter), whereas the scores of the organic field are clustered in the upper left quarter. Glycogen and soluble GST are clearly opposed in B, which is associated to the separation between the high-input and the low-input fields.

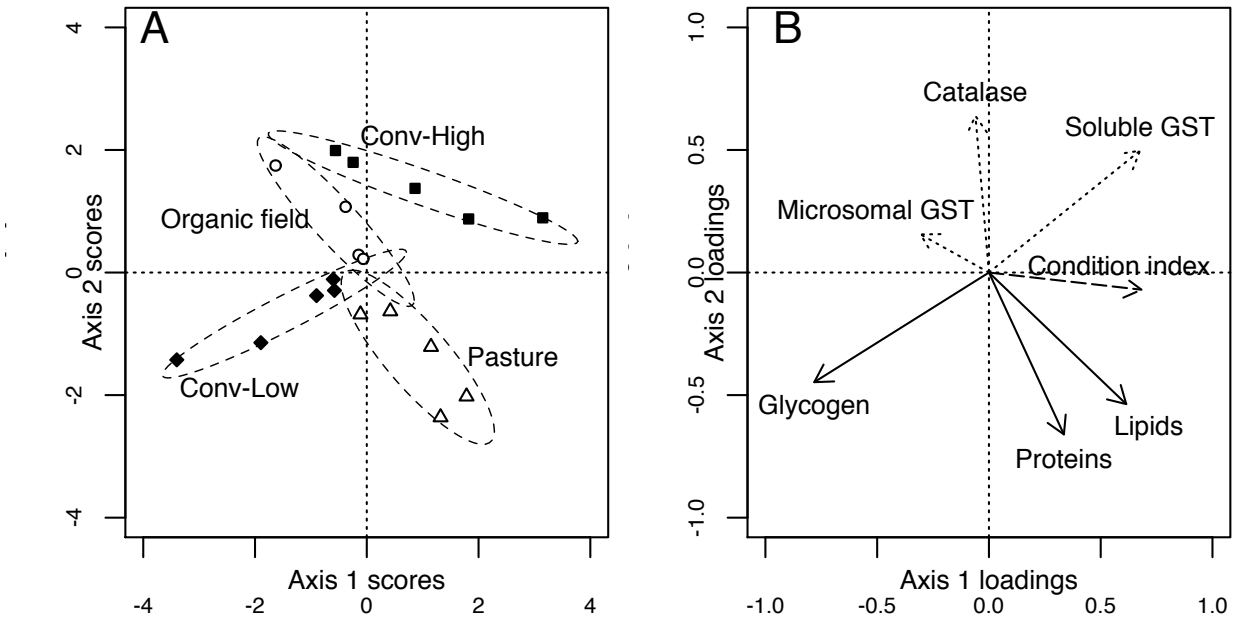

Figure 4: relationships between earthworm biological parameters (7 variables) and field pesticides (5 sampling point per field). Principal components 1 and 2, explaining 30 % respectively 23% of total variability, were kept for interpretation. A: scores plot on Principal Components 1 and 2. Conventional fields are marked with full symbols, and organic field and pasture with empty symbols. Dashed ellipses: 80% confidence. B: variables loadings on Principal Components 1 and 2. Full-lined arrows are energetic parameters, dotted-lined arrows are enzyme activities, and the dashed-line arrow is the condition index.

4. Discussion

4.1. Pesticide residual contamination in agricultural soils

The highest diversity of pesticide molecules was found on the medium input field, followed by the high input, then by the low input. The glyphosate metabolite AMPA was about twice as high in the high input field, compared to the medium, and it was not detected in the low input field. The non-recovery of many of the pesticides recently applied (2009-2010) indicates either degradation, or forming of bound residues (Gevao et al., 2000). This study however highlights the persistency of atrazine, epoxiconazole and alachlore. They have relatively long half-lives in soil: Atrazine 146 days in aerobic soil (PAN Pesticide Database), epoxiconazole a field DT50 > 400 days (Bromilow et al., 1999), and alachlore 20 days in aerobic soil (PAN Pesticide Database). Possibly, anaerobic conditions add to their half-life.

Despite its ban in France in 2003, Atrazine was found in nearly all soil samples of the three conventional fields, and the organic field, but not in the pasture. The pasture and the organic field are located less than one kilometer apart both belonging to the same collective farming group (GAEC La Mandardière) funded in 1992. The pasture had been in this management since 1960 whereas the organic field may have been in conventional agriculture previously as the background before 1992 was not available. This indicates that the residual contamination by atrazine on the organic field is probably not due to spray drift or run-off from other fields nearby, nor rain water (Buser, 1990), but to previous applications before 1992, which possibly makes the residues 20 years old. Capriel *et al* (1985) detected 50% of an initial amount of atrazine 9 years after application. Up to 25% of ^{14}C-labeled atrazine was still present 22 years after the last application on an agricultural soil (Jablonowski et al., 2008). Atrazine persistence in the soils, as confirmed by our results, could explain why it is still found in surface waters in Brittany despite its interdiction in 2003 in France. Still in 2011, ten rivers of Brittany contained the metabolites 2-hydroxy atrazine and atrazine desethyl as the second and third most frequently detected molecules (more than 50% of the water samples), just after the glyphosate metabolite AMPA. The parent compound was only found in 11% of the water samples (CORPEP, 2011). Our study supports that contamination of surface waters by atrazine and its metabolites likely occurs through slow release of the compound from bound residues in agricultural soils (Johnson et al., 1999).

4.2. Comparison of earthworm responses between fields and species

Detoxication capacities of earthworms not only correlated to the residual pesticide concentration between conventional and organic soils, but differed also species specific. Activities of sGST and Catalase were higher with increasing historical inputs of pesticides in *A. caliginosa*, whereas in *A. chlorotica*, only mGST activity followed this trend.

Clear species differences were also shown in the available energy resources. Whereas in *A. chlorotica* all energetic parameters were lower in the conventional fields compared to the pasture, and lipids and proteins lowest at highest pesticide content, in *A. caliginosa*, only the glycogen was that clearly affected. From the whole body energy budged, it can thus be assumed that increasing pesticide contents will have consequences for population fitness, similar to the studies of metal resistance (Pook et al., 2009; Holmstup et al., 2011). Comparing the species, *A. chlorotica* would be more affected.

A. caliginosa from the high-input field were quite distinct from the other fields by Principal Component Analysis due to higher sGST and Catalase activities, as well as low glycogen content. The organic field and the pasture differed mainly according to energetic contents, and Catalase activity. Similarly, different weeding maintenances in vineyards were reflected in enzyme activity changes and even more in life traits (Schreck et al., 2012).

Since the EEC early 1980's recommendation to use the epigeic *Eisenia fetida* as a test species for acute toxicity studies (Edwards, 1984; OECD 1984), several comparative toxicity tests evidenced *E. fetida* to be less sensitive to pollutants than the more ecologically relevant *A. caliginosa* or *L. terrestris* (Pelosi et al., 2013b). Explanations for these sensitivities could be differences in the species' abilities to regulate contaminants uptake, bind xenobiotics or their metabolites, or metabolic rates (Fitzgerald et al., 1996; Gilman and Vardanis, 1974; Pelosi et al., 2013b). As both, *A. caligiosa* and *A. chlorotica* were sensitive to residual contaminations in the complex field situations, they could serve for environmental assessment using enzymatic and energetic biomarkers. More detailed investigations about concentration kinetics and specificity of the physiological responses would be needed.

4.3. Experimental exposure

Epoxiconazole is a large-spectrum fungicide, which acts by inhibiting the enzyme lanosterol 14-α-demethylase (CYP51) controlling the biosynthesis of ergosterol, a constituent of the cellular membrane solely in fungi. It has been shown to affect other cytochrome p450 enzymes and cause endocrine disruption (Taxvig et al., 2007; Kjærstad et al., 2010); it is also suspected of genotoxicity (Akcha et al., 2008). Detoxification via cytochrome p450 followed by GST has been evidenced in rats, hence might occur in other organisms too (Hester et al., 2012). Contrastingly to the pre-adapted population, the naïve one was not able to increase the activity of the sGST and therewith possible detoxication. Schreck *et al* (2008) exposed naïve earthworms *A. caliginosa* to four non-triazole fungicides (folpet, myclobutanil, metalaxyl and fosetyl-Al) and found no significant activation of GST. This is consistent with our results; apparently pre-exposure to the pesticides in the fields is required for activation of the sGST detoxication system of *A. caliginosa.*

Glyphosate is a systemic herbicide acting through enzyme inhibition in the Shikimate pathway which is unique for plants, fungi and bacteria for producing aromatic amino acids, that are then essential for animals (Herrmann and Weaver, 1999). It is thus expected to have little impact on animals. However, adverse effects of glyphosate, particularly as commercial formulations, i.e with surfactants and other compounds added to the active ingredients have been evidenced in animals (Glusczak et al., 2006; Contardo-Jara et al., 2009; Modesto and Martinez, 2010). Commonly in both populations glyphosate alone and the mixture decreased the activity of mGST. In the earthworm *Eisenia fetida andrei* a formulation of Glyphosate applied at recommended rate altered membrane stability as shown by the Neutral Red Retention Time (NRRT) assay (Casabé et al., 2007). Hence the decrease in mGST could be indirectly via membrane derangement by glyphosate and its adjuvants. The detoxication possibility via this enzyme is then non-available. This was not the case for epoxiconazole, which only induced a short-term decrease in mGST, and the activity went back to control level thereafter.

Decrease of catalase activity was observed with a similar pattern in both populations. This could indicate that an oxidative stress occurs, over exceeding the capacity of Catalase and decreasing its activity as shown in the same study by Schreck *et al* (2008). However the measurements of oxidative damage parameters such as lipid peroxidation would be necessary to verify this assumption (Winston and Digiulio, 1991; Modesto and Martinez, 2010).

Conclusions

Pesticide residues in conventional soils and the persistence of several of them leads to their presence years after their last application, possibly affecting soil biota. The agricultural exposure history of earthworms originating from such fields correlated to their basal and inducible detoxication capacities and correlated energy demands. Species differences appeared in the detoxication strategies of field-sampled *Aporrectodea caliginosa* and *Allolobophora chlorotica,* associated with apparent energetic costs in the main energetic reservoirs in *A. chlorotica,* thus may contribute to the decrease of their population density and species biodiversity in cropped fields.

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Supplementary materials

(submitted with the main manuscript)

Materials and methods:

2.2 Pesticides extraction and determination of concentration in the soil

Pesticide extraction and samples preparation:

For the multi-residue extraction, 2g of soil sample were suspended in 500 ml of mineral water by ultrasonic treatment (2x15 min each in a bath) and rotary shaking (one hour). 0.25 µg of triadimenol was added as internal standard and 1 ml of analytical-grade nitric acid (HNO_3) to acidify the mixture. Then pesticides were extracted from the aqueous suspension in two steps with 25 ml of HPLC-grade dichloromethane followed by 15 min of agitation. The dichloromethane extracts were combined and evaporated to a drop (below 10 µl), dissolved in 500 µl acetonitrile and 0.25 µg of pentabromophenol were added as second internal standard. The extract was evaporated again to a drop and adjusted to 500 µl with 10% acetonitrile acidified with 0.1 % formic acid.

For the glyphosate extraction, 0.5 g of soil sample was suspended in 500 ml of mineral water by ultrasound treatment and rotary shaking (as above). 100 ml of sample was transferred in polyethylene flask (250ml), and 50µl (10mg/l) of internal standard was added (glyphosate $^{13}C^{15}N$ and AMPA $^{13}C^{15}N$). The pH was adjusted to 1 with hydrochloric acid, mixed for one minute than neutralize by 4.6 ml of KOH 3M. Sample was derivatized by adding 5mL of borate buffer ($Na_2B_4O_7$, $10H_2O$, 84mM) and 10ml of FMOC-Cl solution (3.2mM) by agitation for 2 h at room temperature. Water samples were then extracted by solid-phase extraction (OASIS HLB Cartridges, 6cc, 0.2g (WATERS) with ASPEC XL (Gilson). Analytes were eluted by acetate ammonium/acetonitril (50/50) and conserved at -18°C. Excess derivatization reagent was eliminated by washing sample with 2 mL of diethyl ether and then concentrated to 1 ml and homogenized in ultrasonic bath.

Analytical procedures:

Samples for both multi-residue and glyphosate were analysed by LC/MS: Waters Alliance 2695 separations module coupled to a Waters Micromass ZQ mass spectrometer (Waters, St Quentin en Yvelines, France) equipped with the Z-Spray™ electrospray ionization (ESI)

source. Chromatographic separations were performed on a Waters XTerra® MS C18 column (150 mm length x 2.1 mm I.D., 3.5 µm particle size), at flow rates of 0.2 $mL.min^{-1}$ and 0.25 $mL.min^{-1}$, and maintained at constant temperatures of 35 and 40°C, for the multi-residue and the glyphosate method, respectively. For monitored ions, cone voltages and ion mode, see table S3.

Analytical conditions for the multi-residue method:

The binary mobile phase was composed of ultra-pure water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid. The sample injection volume was 10 µL. Samples were dissolved in mobile phase at initial conditions (82% A) prior to injection. The gradient elution program was as follows: 0-10 min, 82-70% A; 10-20 min, 70-50% A; 20-35 min, 50-20% A; 35-40 min, 20% A; 40-40.1 min, 20-82% A (return to initial conditions); 40.1-50 min, 82% A (equilibration). A standard curve was made with several aliquots of 2 g of dry soil (uncontaminated soil from the pasture) spiked manually with the purified compounds, air-dried for two hours, and extracted the same way as the real samples for quantification, relating to the two internal standards.

Analytical conditions for glyphosate-AMPA:

The binary mobile phase was composed of ammonium acetate 5mM (solvent A) and acetonitrile (solvent B). The sample injection volume was 20 µL. The gradient elution program was as follows: 0-3 min, 90-75% A; 3-13 min, 75-70% A; 13-18 min, 70-5% A; 18-26 min, 5% A; 26-28 min, 5-90% A (return to initial conditions); 28-45 min, 90% A (equilibration). Quantification was done relating to a calibration curve of the pure glyphosate or its metabolite AMPA in 100 ml of mineral water extracted the same way as the samples and relating to the internal standards. Pesticides analytical standards were purchased from Dr Ehrenstorfer (Ausburg, Germany).

2.5. Energy resources measurements

Lipids were extracted according to Folch (1957), and assayed by the sulfo-vanillin method with a calibration curve of commercial vegetable oil at 525 nm. Protein sample aliquots were

homogenized in 0.1 M phosphate buffer (pH 6.5), centrifuged and soluble protein content measured in the supernatant according to Bradford (1976) as above. Glycogen was measured according to the method of Nicolai et al. (2012) adapted to a microplate reader (Fischer Scientific Multiskan FC). Briefly, aliquots were homogenized in 600 µl trichloroacetic acid (4%) and centrifuged at 5000xg for 10 minutes. To 500 µl of supernatant 1.5 ml of ethanol was added and stirring for 10 minutes precipitated glycogen. The extract was centrifuged (5000xg) and the ethanol eliminated with a glass pipette. The washing was repeated once, then the remaining ethanol was totally evaporated at 70°C. The pellet was redissolved by stirring it overnight in ultrapure water, and centrifuged again. Absorbance was measured at 425 nm after addition of Lugol using a calibration curve of purified glycogen (Oyster type II, Sigma).

Table S1: Soil texture and chemical characteristics of the five fields studied from agricultural landscapes.

	Clay %	Silt %	Sand %	Organic matter %	pH (water suspension)	CEC cmolc/ kg	Cu (EDTA extracted – (mg/kg)	Zn (EDTA extracted - mg/kg)	Ca (total) %	K (total) %
Pasture	17.6	69.3	13.1	4.03	6.03	9.21	14.2	7.55	0.3	1.48
Organic field	16.6	71.0	12.4	2.55	6.95	8.46	17.5	9.71	0.36	1.51
Low-input	19.5	59.4	21.1	3.1	6.93	7.92	13.7	6.48	0.32	1.35
Average-input	15.7	69.9	14..4	2.06	6.63	6.39	17	7.12	0.3	1.43
High-input	14.8	71.6	13.6	1.67	6.39	5.48	16.7	6.48	0.33	1.34

Table S2: pesticides molecules analysed in the soil, effect, mean recovery rate (out of 5 extractions and detection limits.

Active molecule	**action**	**Detection limit ($\mu g.g^{-1}$ dry soil)**	**Mean recovery rate (mean of 5 replicates [SD])**
Pesticides applied between 2005 and 2011 (17)			
alachlore	herbicide	0.8	81% ± [9%]
met: CDEPA	metabolite	1.7	97% ± [10%]
azoxystrobine	herbicide	4.2	98% ± [7%]
bentazone	herbicide	4.2	106% ± [4%]
boscalid	fungicide	0.8	90% ± [8%]
chlortoluron	herbicide	1.7	91% ± [6%]
met: IPPMU	metabolite	0.8	98% ± [5%]
cyprodinyl	fungicide	0.8	95% ± [14%]
dicamba	herbicide	0.8	97% ± [1%]
epoxiconazole	fungicide	0.8	75% ± [5%]
glyphosate	herbicide	50	91%
met : AMPA	metabolite	50	56%
ioxynil	herbicide	4.2	158% ± [16%]
mesotrione	herbicide	4.2	87% ± [12%]
metazachlore	herbicide	5.8	86% ± [12%]
metolachlore	herbicide	1.7	81% ± [8%]
nicosulfuron	herbicide	1.7	126% ± [5%]
sulcotrione	herbicide	1.7	98% ± [5%]
tebuconazole	fungicide	1.7	76% ± [6%]
Active molecules applied between 2001 and 2005 (5)			
mecoprop	herbicide	0.8	88% ± [6%]
linuron	herbicide	1.7	89% ± [4%]
oxadixyl	fungicide	4.2	89% ± [5%]

simazine	herbicide	0.4	84%	±	[4%]
trichlopyr	herbicide	0.8	78%	±	[8%]
Active molecules non applied between 2001 and 2011 (19)					
isoxaflutole	herbicide	0.8	127%	±	[18%]
metosulam	herbicide	4.2	106%	±	[3%]
isoxaben	herbicide	1.7	91%	±	[5%]
propachlor	herbicide	0.8	101%	±	[10%]
propiconazole	fungicide	1.7	70%	±	[7%]
propyzamide	herbicide	1.7	87%	±	[5%]
tebutame	herbicide	0.8	94%	±	[12%]
terbumeton	herbicide	0.4	83%	±	[9%]
terbutylazine	herbicide	0.8	78%	±	[8%]
met: desethyl terbutylazine	metabolite	0.4	95%	±	[4%]
tetraconazole	fungicide	4.2	79%	±	[7%]
thiametoxam	insecticide	4.2	90%	±	[5%]
atrazine	herbicide	0.4	83%	±	[6%]
bromoxinyl	herbicide	0.8	114%	±	[7%]
carbofuran	insecticide	1.7	92%	±	[7%]
dichlorprop	herbicide	1.7	133%	±	[18%]
diuron	herbicide	5.8	100%	±	[7%]

Table S3: analytical characteristics of the measured compounds (monitored ions, cone voltages and ion mode)

Compound	**CAS number**	**Chemical family**	**Ion 1**	**Ion 2**	**Cone voltage (V) ion 1/ion 2**	**Ion mode ion 1/ion 2**
Bentazone	25057-89-0	Benzothiadiazinones	197.1	239.1	60/45	ES-/ES-
Bromoxynil	1689-84-5	Hydroxybenzonitriles	273.9	276.0	60/60	ES-/ES-
Dicamba	1918-00-9	Benzoic acids	219.1	221.1	15/15	ES-/ES-
Ioxynil	1689-83-4	Hydroxybenzonitriles	126.9	369.9	60/15	ES-/ES-
MCPA	94-74-6	Phenoxy carboxylic acids	141.2	199.2	45/25	ES-/ES-
Dichlorprop	120-36-5	Phenoxy carboxylic acids	161.0	233.1	45/25	ES-/ES-
Mecoprop	7085-19-0	Phenoxy carboxylic acids	141.1	143.1	45/60	ES-/ES-
Trichlopyr	55335-06-3	Pyridine carboxylic acids	196.0	256.0	40/15	ES-/ES-
Mesotrione	104206-82-8	Triketones	290.9	337.9	40/18	ES-/ES-
Metosulam	139528-85-1	Triazolopyrimidines	175.2	418.2	60/45	ES+/ES+
Nicosulfuron	111991-09-4	Sulfonylureas	182.3	411.2	60/25	ES+/ES+
Sulcotrione	99105-77-8	Triketones	139.0	328.9	60/45	ES+/ES+
Desethyl terbutylazine	30125-63-4	Triazines	202.0	204.0	25/25	ES+/ES+
Simazine	122-34-9	Triazines	104.0	202.2	60/45	ES+/ES+
Terbutylazine	5915-41-3	Triazines	230.0	232.0	25/25	ES+/ES+
Terbumeton	33693-04-8	Triazines	226.1	227.1	25/25	ES+/ES+
Chlortoluron	15545-48-9	Substituted ureas	213.2	215.1	25/25	ES+/ES+
Diuron	330-54-1	Substituted ureas	231.1	233.1	25/25	ES-/ES-
Epoxiconazole	133855-98-8	Triazoles	329.9	331.8	15/15	ES+/ES+
Propiconazole	60207-90-1	Triazoles	159.1	342.0	70/38	ES+/ES+
Tetraconazole	112281-77-3	Triazoles	159.2	372.1	60/45	ES+/ES+
Tebuconazole	107534-96-3	Triazoles	308.3	310.3	25/25	ES+/ES+
Alachlor	15972-60-8	Chloroacetamides	162.3	238.2	60/25	ES+/ES+
Oxadixyl	77732-09-3	Phenylamides	219.0	279.0	30/15	ES+/ES+
Metolachlor	51218-45-2	Chloroacetamides	176.3	284.2	60/25	ES+/ES+
Metazachlor	67129-08-2	Chloroacetamides	210.0	278.0	25/25	ES+/ES+
Tebutam	35256-85-0	Benzamides	192.3	234.4	50/15	ES+/ES+
Azoxystrobin	131860-33-8	Strobilurins	344.1	372.1	60/45	ES+/ES+

Boscalid	188425-85-6	Carboxamides	140.0	342.8	55/30	ES+/ES+
Carbofuran	1563-66-2	Carbamates	123.1	165.2	60/45	ES+/ES+
Cyprodinil	121552-61-2	Anilinopyrimidines	226.3	227.3	60/60	ES+/ES+
Isoxaben	82558-50-7	Benzamides	165.1	333.1	50/25	ES+/ES+
Atrazine	1912-24-9	Triazines	174.1	216.2	45/25	ES+/ES+
Glyphosate	1071-83-6	Organophosphorous	150	168	60/45	ES-/ES-
AMPA	77521-29-0		332	110	15/35	ES-/ES-
Glyphosate $^{13}C^{15}N$			395.1	153.1	25/60	ES+/ES-
AMPA $^{13}C^{15}N$			335.1	137.0	25/45	ES+/ES-_

Table S4: initial weights and weight changes along the study of pre-exposed and naïve earthworms *A. caliginosa* (sampled in the spring of 2012) after 3, 7 and 28 days. Values are mean out of 6 replicates (N=6) ± *standard deviation* (SD) per group. Significant weight losses throughout the study were indicated with an * (one way anova with time as factor for each soil treatment followed by Tukey's post-hoc test).

Population	Initial weight (T0) g FW	Soil Treatment	Weight change after exposure in % initial weight									
			Day 3			Day 7			Day 28			
Conventional	0.32 ± 0.06	Control	97%	±	9	89%	±	12	83%	±	13	
		Fungicide-treated	94%	±	13	102%	±	10	96%	±	29	
		Herbicide-treated	98%	±	9	101%	±	9	77%	±	7	*
		Mixture-treated	97%	±	20	96%	±	17	83%	±	13	
Organic field	0.69 ± 0.15	Control	100%	±	9	86%	±	10	81%	±	14	
		Fungicide-treated	95%	±	12	86%	±	9	79%	±	11	
		Herbicide-treated	95%	±	23	82%	±	10	82%	±	10	
		Mixture-treated	87%	±	7	81%	±	6	78%	±	4	*

Manuscript 2:

ACCLIMATION OF EARTHWORMS TO CHEMICALS IN ANTHROPOGENIC LANDSCAPES, PHYSIOLOGICAL MECHANISMS AND SOIL ECOLOGICAL IMPLICATIONS

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Acclimation of earthworms to chemicals in anthropogenic landscapes, physiological mechanisms and soil ecological implications

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Summary

Because earthworms sustain soil functioning and fertility, there is a need to advance the knowledge of their adaptation potential to chemicals in anthropogenic landscapes. Our hypothesis is that there is acclimation to organic chemicals (pesticides) in earthworms that durably persist under conventional farming in anthropogenic landscapes. The adaptation capability of two populations of earthworms *(Aporectodea caliginosa)* having a different chemical exposure history, - one originating from 20 years of organic farming (naïve population) and another from 20 years of conventional farming (pre-exposed population)- to cope with soil organic pollutant (Opus®, epoxiconazole a worldwide used fungicide) were investigated. Several complementary metabolic and energetic endpoints were followed, and cast production was assessed as a behavioral biomarker related to earthworms ecological role for the soil. Basal metabolism reflected by respiration rate was increased in both fungicide-exposed worms compared to controls. Glycogen resources were decreased in the same proportion in the two populations but more rapidly for the naïve (7 days) than for the pre-exposed population (28 days). Soluble protein and most amino-acids contents increased in the pre-exposed population only, suggesting a detoxification mechanism. Metabolomic profiles showed a cut-off between fungicide-exposed and control groups in the pre-exposed earthworms only, with an increase in most of the metabolites. Exposure to a low dose of epoxiconazole increased cast production of pre-exposed earthworms, and this resulted in an increase in pesticide disappearance. As far as we know, this is the first study which evidenced there is an acclimation to an agricultural chemical in earthworms derived from conventional farming that also relates to a change in their burrowing behaviour, and for which larger consequences for the soil ecosystem need to be addressed. This original finding is of major interest in the frame of ecosystem resilience to global changes. Whether this physiological adaptation is a general pattern of response against fungicides or other pesticides would need to be confirmed with other molecules and agricultural contexts.

Keywords

Land-use, Epoxiconazole, Earthworm adaptation, Energy storage, Metabolomic profile, Soil bioturbation

1. Introduction

Often representing the largest animal biomass, earthworms are present in most terrestrial ecosystems and are considered as efficient ecosystem engineers as they actively modify the physical, chemical and biological properties of the soil (Binet et al., 1998; Bottinelli et al., 2010; Jones et al., 1997; Monard et al., 2008). They sustain several key ecosystem services by enhancing soil structure and nutrient cycling, and play a role in ecosytemic services such as water regulation, pollution remediation and primary production (Blouin et al., 2013). In anthropogenic landscapes, soil biodiversity and mainly earthworm communities have to face disturbances by intensive land-use due to agricultural practices such as ploughing and tillage, application of fertilizers and chemical pesticides (Paoletti, 1999; Thompson, 1971). The soil compartment is the primary sink for agricultural pesticides, as they are frequently applied several times a year. Concerning fungicides, a large part either do not reach its plant target, or is washed off from treated foliage, leading to major losses to the soil. Bromilow *et al* (1999a) reported in a field study that only 30% of the fungicide sprays were intercepted by the barley crop. The frequent application of agricultural pesticides and the persistence of some of them eventually leads to increasing amounts of residual compounds in the soil, either as free or bound residues (Gevao et al., 2000; Mordaunt et al., 2005), which can be a threat to lumbricids species. It has been shown that abundance and diversity of earthworm communities are dramatically reduced by chemical and mechanical stress (Decaëns and Jiménez, 2002; Smith et al., 2008). Despite these impacts however, worm populations persist in conventionally (with pesticides usage) cropped fields, albeit in reduced numbers. Assuming that immigration rate is low in earthworm populations (Lavelle and Spain, 2001), this suggests that earthworm populations living in pesticide-polluted soils can cope with chronic chemical stress, either via avoidance behaviour or physiological resistance (Posthuma and Van Straalen, 1993).

A chronic exposure to contaminants for generations may allow adaptation to take place, by favouring individuals that are able to face them. Physiological adaptation, e.g. acclimation, implies that individuals have acquired a degree of tolerance after a pre-exposure to chemicals at some point of their life, which can be lost within a generation. Genetic adaptation to xenobiotics implies constitutive and hereditary mechanisms allowing tolerance such as overproduction of specific compounds, (Brausch and Smith, 2009), or alteration of a target or

receptor (Fournier and Mutero, 1994). Such adaptations to metals have been studied in terrestrial invertebrates including earthworms (Donker et al., 1993; Gudbrandsen et al., 2007; Posthuma, 1990). However to the extent of our knowledge no attempt has been made to evaluate adaptation processes in earthworms against organic pesticides.

Adaptation, either via physiological or genetically-mediated mechanisms, is an expression of the species ecological plasticity allowing protection against stresses. However, it is known to be costly in terms of metabolism and energy usage, especially when it involves over-production of compounds such as protection enzymes (Calow, 1991). Increased metabolic rate or increased energy allocation to detoxification mechanisms can be at the detriment of energy storage, and thus impair other functions such as reproduction and growth (Jansen et al., 2011; Yasmin and D'Souza, 2010). This is particularly important when animals are exposed to low but chronic exposures, such as earthworms in agricultural fields, due to the persistence of residues of pesticides in the soils for several years after application (Gevao et al., 2000). Available energy resources, as measured by main storage compounds, can then reflect energy demands associated with different adaptation strategies. In the earthworm *Dendrobaena octaedra*, Holmstrup *et al* (2011) reported high energetic costs reflected by glycogen depletion of the internal regulation of Al and Ni metals. On a lower level of organization, and possibly on a shorter timescale, metabolomics have recently been used in ecotoxicology studies to investigate the responses of the metabolism to contaminants (Brown et al., 2010; Bundy et al., 2008; Simpson and McKelvie, 2009), and we believe they can be a valuable tool to study adaptation to contaminants.

One of the most common cultures in Brittany (France) is winter wheat, with a mean number of 6 pesticides applications per year, the majority of them being fungicides (Agreste, 2006). In particular, members of the triazole family, which act by inhibiting the biosynthesis of ergosterol, have attracted interest because of their high persistency in soils (Bromilow et al., 1999a, 1999b; Passeport et al., 2011), although data available on their toxicity is still scarce.

The toxicity of some fungicides, such as benomyl and carbendazim has been investigated in a few studies in earthworms and enchytraeids, another important member of the soil biocenosis. Avoidance behaviour was reported in Enchytraeids following exposure to benomyl and Carbendazim (Amorim et al., 2005). Holmstrup (2000) also reported a decrease of

reproductive rate in the earthworm *Aporrectodea longa* associated with a reduction in the whole earthworm population.

Our aim was to test the hypothesis that earthworms inhabiting soil under conventional farming have acquired tolerance to face the regularly applied fungicide epoxiconazole. The response of in situ pre-exposed versus naïve earthworms when exposed to an environmentally relevant dose of the fungicide were thus studied. We addressed whether an adaptation was quantifiable in terms of main energy resources storage and metabolism (respiration and metabolites levels) by comparing these two pre-exposed and naïve earthworms, and if these physiological changes were associated with change of their soil bioturbation ability. Our results evidenced that there is a physiological adaptation to fungicide in the earthworms originated from the conventional cropped field, leading to a change in their burrowing behaviour and impacting the fate of pesticide in soil.

2. Materials and Methods

2.1. Earthworm populations, soil and agricultural context

Earthworms used in this study originated from two agricultural fields, one conventionally cropped and one cropped according to organic agriculture requirements. Both of these fields have been in these agricultural management strategies for more than 20 years and are located in the same agricultural basin (Vézin-le-Coquet, Britanny, France). Soils are slightly acid silt-clay loams (conventional and organic field, respectively: Clay 14.8 % and 16.6 %; Silt 71.6 % and 71%; Sand 13.6 % and 12.4 %; organic matter 1.67 % and 2.55 %; pH (water suspension) 6.4 and 6.9). The conventional field had been cropped under rotations of wheat/maize/leguminous for 20 years, and annually treated with pesticides. The fungicide epoxiconazole was used each year a cereal was planted. Epoxiconazole is a triazole fungicide present in two pesticides formulations (OPUS® and OGAM®) and mainly used on wheat. The organic field has been under rotation with a cereal (2 years) / maize (1year) /lucerne (3 years) without any pesticides for 20 years, and was not tilled during the lucerne periods.

Soil used for the exposure experiment was collected from the first 30cm of a permanent (since 1960) organic pasture (17.6 % clay, 69.3 % silt, 13.1 % sand, 4.0 % organic matter, pH (water suspension) 6.0) located in the same area where no initial epoxiconazole residuals were detected (see method in 2.3). Upon retrieval, it was air-dried until it reached 14 % of humidity, then sieved to remove all soil particles larger than 2mm and kept in sealed containers (100 l) until used for the experiment.

The endogeic species *Aporretodea caliginosa* was chosen for this experiment. It is an environmentally relevant species for toxicological tests, since it is commonly found in agricultural fields and reported as a dominant species (Jordan et al., 2004; Lamandé et al., 2003; Nuutinen, 1992; Söchtig and Larink, 1992). Most standard ecotoxicity tests are conducted on epigeic species mainly with *Eisenia fetida* or *Eisenia andrei*, but these species lack ecological relevance since they are usually absent from agricultural fields (Dittbrenner et al., 2010). Earthworms were collected by hand-sorting at the beginning of Spring 2012 from the two fields. Adults (presence of a fully developed clitellum) and sub-adults (presence of tubercula pubertatis) were used and individual weights were recorded (Table 1). Upon collection, they were brought back to the laboratory and maintained in the soil collected from the field at 25 % humidity until start of fungicide exposure. Before the experiment, the earthworms were acclimatized for 14 days in the test soil in the climatic room used for the experiment (Conviron GR96; temperature: 15 °C; day/night cycle: 16/8 h; humidity: 80 ± 5%).

2.2. Experimental setup

Soil contamination: Epoxiconazole was applied as commercial formulation OPUS® (125 g active ingredient l^{-1}, obtained from BayerCropScience) diluted in distilled water at 0.1 <mu>g g^{-1} soil, which is equivalent to a predicted field concentration calculated for a field application rate of 125 $g.ha^{-1}$ assuming a single application with an homogenous distribution and no crop interception in the top 5 cm of the soil (Dittbrenner et al., 2010). Soil spiking was conducted by manually adding 175 ml of the diluted pesticide solution or distilled water (for the controls) on each 2 kg of soil at 14% water content (1.75 kg dry weight) reaching a final soil water content of 24%. To insure homogeneity of pesticide distribution in the soil, the solution was added in two parts, the soil being thoroughly mixed, resieved using a 2 mm aperture and

redisposed as a fine layer. Soil microcosms consisted of polycarbonate boxes (80mm x 50mm, Caubère, Yebles, France) with a lid pierced with tiny holes to ensure sufficient aeration. The microcosms were filled with 100 g of contaminated or control soil, then 0.2 g of dry grass meal was added to the surface of the soil. Then the microcosms were left two days in a cool dark room to ensure aeration of the soil after re-humidification. Water content was checked again in 3 additional control boxes and adjusted to 25% prior to introduction of animals, then checked again each week.

Experimental design: The experimental design is described in table 1. It comprised 11 specimens from each population (pre-exposed and naïve) for each treatment (epoxiconazole or control) and sampling time (7 and 28 days) plus an initial control group at day 0 (unexposed). Prior start of exposure, each earthworm was rinsed in tap water, gently dried on filter paper, weighed and placed in individual Petri dishes for 48 hours for gut voiding. Then animals were transferred individually to the exposure microcosms (day 0) according to a size-class procedure, insuring a similar mean earthworm weight in each treatment. Soil was spiked with epoxiconazole at 0.15 $\mu g.g^{-1}$ soil or control soil. Exposure lasted for 7 and 28 days, with an initial control group of 11 individuals for each population (unexposed worms) at day 0. At each sampling date, before use of the microcosms for cast production (see 2.5) and pesticide measurements (see 2.3), earthworms were removed from the soil taking care not to break the casts. 5 of them were used for respirometry assessment (2.4), energy resources (2.6), and metabolites measurements (2.7). The other 6 earthworms were used for enzyme activities measurements in another study and will not be considered here. After the worms have been sampled, 8 microcosms were used for cast production measurements, and 3 for pesticide analysis.

In addition, eight uncontaminated soil microcosms without worms were used as controls for the cast production test to assess the potential formation of non-biogenic aggregates at 7 and 28 days. Another 3 soil microcosms were filled with contaminated soil to track the fate of pesticide without the presence of worms. Humidity control was conducted in 3 additional, non-contaminated soil microcosms, in order to adjust humidity when necessary.

Table 1: experimental design of the laboratory exposure. Out of each group of 11 microcosms containing one earthworm, 5 randomly picked worms were used for respirometry assessment, energy resources, and metabolite measurements.

		worms at start	Sampling days and number of worms sampled			treatment
			0	7	28	
Microscosms containing one earthworm	Pre-exposed population (conventional field)	33	11	11	11	CTRL
		22	0	11	11	EPOXI
	"Naïve" population (organic field)	33	11	11	11	CTRL
		22	0	11	11	EPOXI
Microcosms without earthworm	Control microcosms for cast production	0	8	8	8	CTRL
	Control microcosms for pesticide dissipation	0	3	3	3	EPOXI
	Control microcosms for humidity check	0	3	3	3	CTRL

2.3. Pesticide concentration in soil

Sub-samples of 2g of soil were retrieved the day of the pollution (day 0), and after 7 and 28 days, from three randomly chosen microscoms out of the 11 replicates. They were dried at 30°C overnight, then kept frozen until pesticide analysis. Epoxiconazole in soil sub-samples was measured by liquid chromatography coupled with mass spectrometer (LC-MS) (Waters alliance 2690, Waters, Saint Quentin en Yvelines, France). 2g of soil sample were extracted in 500 ml of mineral water. After 15 min of ultrasound treatment, the sample was mixed by rotary shaking for one hour, and subjected to another 15 min of ultrasound treatment. 0.25 µg of triadimenol was added as internal standard and 1 ml of analytical-grade nitric acid (HNO_3) to acidify the mixture. Then pesticides were extracted from the aqueous mixture in two steps with 25 ml of HPLC-grade dichloromethane followed by 15 min of agitation. The dichloromethane extracts were combined and evaporated to a drop (ca. 10 µl), then 500 µl acetonitrile were added and 0.25 µg of pentabromophenol were added as second internal

standard. The extract was evaporated again to a drop and adjusted to 500 µl with 10% acetonitrile acidified (0.1 % formic acid).

This sample was analysed by LC-MS using a high-performance liquid chromatography (Alliance 2695, Waters, Saint Quentin en Yvelines, France) coupled to a quadrupole mass spectrometer model ZQ (Waters-Micromass, Saint Quentin en Yvelines, France) equipped with an electrospray source. Epoxiconazole was separated on a X Terra MS C18 column (150 x 2.1 mm, 3.5 mm particle size, Waters, Saint Quentin en Yvelines, France) at 35 °C. A binary mobile phase gradient (A: ultrapure water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid) was used for pesticide separation. The chromatographic method held the initial mobile phase composition (82% A, 18% B) constant for 10 min, followed by 70% A / 30% B (10min), 50% A / 50% B (15 min), 20% A-80% B (5 min), then again 82% A / 18% B for 10 min. Quantification limit was 2.5 $ng.g^{-1}$ dry soil and extraction yield was 75 ± 7%. A standard curve of epoxiconazole was made with several aliquots of 2 g of dry soil (the same uncontaminated pasture soil used for the microcosms) spiked manually with the purified compound, air-dried for two hours, and extracted the same way as the real samples for quantification, relating to the two internal standards. Pesticides analytical standards were purchased from Dr Ehrenstorfer (Ausburg, Germany).

Recovery of epoxiconazole in spiked soil was initially 80% of the desired concentration (0.1 $\mu g.g^{-1}$), with a coefficient of variation of 10%, which was considered highly satisfactory, as we used the commercial formulation of the pesticide. Concentrations of epoxiconazole in soil (Table 2) were still two thirds of the initial concentration after 28 days, which is consistent with the long persistencies (half-life > two years) reported in the literature (Bromilow et al., 1999a, 1999b; Liang et al., 2012).

2.4. Respirometry measurements

After 7 and 28 days of exposure, earthworms were removed from the soil microcosm, rinsed, gently blotted dry on filter paper, weighed and left 24 hours in a 250 ml glass jar on a moist filter paper for gut voiding. Thereafter, the glass jar was hermetically closed for two hours, and CO_2 was measured by a Micro-Gas Chromatograph (3000A, SRA Instruments) equipped

with a single capillary column Poraplot U and coupled with a thermal conductivity detector. Then the worm was frozen in liquid nitrogen for further measurements of energy resources (2.6) and metabolomics (2.7).

2.5. Cast production

Cast production was measured in the soil of 8 microcosms out of the 11 replicates. The cast production test was conducted according to the protocol of Capowiez et al. (2010) but using a sieve of mesh size 2mm. Soil of the microcosms were dried at 40°C overnight, and sieved by shaking the sieve consistently for 10 s.

2.6. Energy resources measurement

Frozen worms were freeze-dried, and ground to a fine powder by multiple 30 sec agitations with inox beads in 2 ml test tubes in a bead-beater (Retsch MM400, Retsch GbmH, Haan, Germany). Each ground sample was separated in several aliquots, 2 mg for total lipids, 5 mg for soluble proteins, and 2 x 10 mg for glycogen and metabolites measurements. Lipid aliquots were extracted according to Folch (1957), lipids in the chloroform extract were assayed by the sulfo-vanillin method with a calibration curve of commercial vegetable oil at 525 nm. Proteins aliquots were homogenized in 0.1 M phosphate buffer (pH 6.5), centrifuged and measured according to Bradford (1976) using a calibration curve of bovine serum albumin. Glycogen was measured according to the method of Nicolaï *et al* (2012). Briefly, aliquots were homogenized in 600 µl trichloroacetic acid (4%) and centrifuged at 5000 G (rotor N° 12145, SIGMA, 8000 rpm) for 10 minutes. Then 500 µl of supernatant was recovered and glycogen was precipitated by adding 1.5 ml of ethanol and stirring for 10 minutes. The extract was centrifuged (5000 G) and the ethanol eliminated with a glass pipette. The pellet was washed with 2ml ethanol, then the remaining ethanol was totally evaporated at 70°C. The pellet was redissolved by stirring it overnight in ultrapure water, and centrifuged again. Absorbance was measured at 425 nm with a microplate reader (Fischer Scientific

Multiskan FC) after addition of Lugol using a calibration curve of purified glycogen (Oyster type II, Sigma).

2.7. Metabolomics

2.7.1. Sample extraction and derivatization

Metabolite extraction was conducted according to Khodayari *et al* (2013). The freeze-dried and ground sample was homogenized in 600 µL of cold (−20 °C) methanol–chloroform (2:1) using a bead-beating device (Retsch MM301, Retsch GbmH, Haan, Germany). 400 µL of ice-cold ultrapure water was subsequently added, and each sample was stirred. After centrifugation at 4000 G for 10 min at 4 °C, 300 µl of the upper aqueous phase, containing polar metabolites, were transferred to new chromatographic vials and vacuum-dried using a Speed Vac Concentrator (MiVac, Genevac Ltd., Ipswitch, England). The dried extracts were then redissolved in 15 µL of 20 $mg.mL^{-1}$ methoxyaminehydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in pyridine, incubated under automatic orbital shaking at 40°C for 90 min prior to derivatization. Then, 15 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Sigma) was added, and derivatization was conducted at 40 °C for 45 min under agitation. The derivatization process was automatized using a CTC CombiPal autosampler (GERSTEL GmbH and Co.K.G, Mülheim an der Ruhr, Germany).

2.7.2. GC-MS analyses

Gas chromatography coupled with mass spectrometry (GC–MS) was used to measure up to 58 small metabolites belonging to different classes of molecules: amino-acids, polyols, sugars, intermediates of the citric acid cycle and other unclassified biological molecules. The GC–MS system was comprised of a Trace GC Ultra chromatograph, and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA, USA). The injector temperature was set at 250°C. The oven temperature was increased from 70°C to 170°C at 5°C min^{-1}, from 170 to 280°C at 7°C min^{-1}, from 280 to 320°C at 15°C min^{-1}, then the oven remained for 4 min at 320°C. A 30 m fused silica column (TR5 MS, I.D. 2.5 mm,

95% dimethyl siloxane, 5% Phenyl Polysilphenylene–siloxane) was used, with helium as the carrier gas at a rate of 1 $mL.min^{-1}$. One microliter of each sample was injected using the split mode (split ratio: 25:1). We completely randomized the injection of the samples. The temperature of the ion source was set at 250°C and the MS transfer line at 300°C. Detection was achieved using MS detection in electronic impact (EI). We used the selective ion-monitoring mode (SIM) (electron energy: -70 eV), allowing a precise annotation of the detected peaks. The peaks were identified according to both their mass spectra (two ions) and their retention times. Metabolite levels were quantified, if above their quantification limits, according to calibration curves made with 58 pure reference compounds, including the internal standard. Chromatograms were integrated using XCalibur v2.0.7 software (Thermo Fischer Scientific Inc, Waltham, MA, USA).

2.8. Statistical analyses

For both populations, the effect of epoxiconazole compared to control groups, or mean differences between populations, either on respiration, energy storage or cast production was tested by student-t-tests at each sampling time. The disappearance of pesticide in the soil was tested by one-way ANOVA with time as factor for soils containing pre-exposed, naïve and no earthworms, followed by post-hoc tests according to the Tukey procedure. On the metabolomics data, two principal component analyses (PCA) were performed on each population as separate datasets on log-transformed and standardized variables. Three axes, explaining 70% of variability, were kept for interpretation. The variables Serine and Threonine were highly correlated (94%) so their arithmetic sum was used in a single variable, so as not to hamper the results of the PCAs. A classification of the metabolites into "functional biochemical groups" was done following Bundy *et al* (2008) and based on biochemical knowledge. Amino-acids were classified either as lipophilic, hydrophilic or neutral. Regarding the metabolite functional group responses to epoxiconazole, significant differences between control and exposed worms for each metabolite were tested by student-t-tests at 7 and 28 days. Significance level for student-t-tests was set at $p \leq 0.1$. All analyses were conducted using the statistical software of "R 2.12.1" for Macintosh (R Development Core Team, 2008).

3. Results

3.1. Energy dissipation

Fungicide treatment increased CO_2 production in both pre-exposed and naïve populations after 7 and 28 days compared to their non-treated controls (Fig 1). Respiration rate in the fungicide-treated group was higher after 7 and 28 days compared to day 0 in the pre-exposed population. Differences became significant between populations at 28 days, where metabolic rates of the pre-exposed population (both exposed and control groups) were still higher than at the outset, while it remained constant in the naïve population from day 7 to 28.

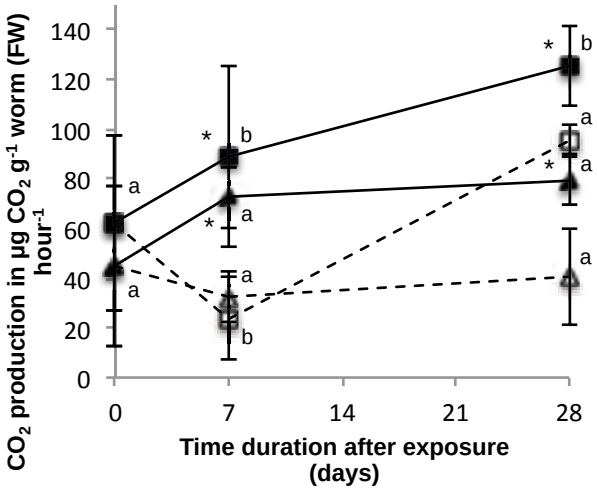

Fig1

Metabolic rate (µg CO_2 g^{-1} worm (fresh weight) $hour^{-1}$) of pre-exposed (*square*) and naïve (*triangle*) *A. caliginosa* exposed to Epoxiconazole (*solid symbols, full lines)* or not exposed (o*pen symbols, dashed lines*) at day 0, 7 and 28. Mean values (N=5 worms per group) are presented with standard deviations as error bars. Significant differences are indicated with * between exposed and control worms and # between populations at each sampling time. Different letters (a or b) denote statistical differences between sampling times within the same group.

3.2. Cast production (CP)

The weight of casts (g of dry cast day^{-1}) correlated linearly and positively to earthworm weights at 7 and 28 days (R^2 =0.34; $p<0.001$ and R^2=0.33; $p<0.05$, respectively, all modalities

mixed). The cast production was then calculated as weight difference of non-biogenic aggregates retained in the sieve from the 8 control (without worms) microcosms to the ones with worms and expressed per gram of fresh worm. Worms from both populations displayed similar patterns with constant CP over time in control soil microcosm and significantly changed CP with fungicide treatment (Figure 2). Application of epoxiconazole transitory enhanced CP (7 days) in pre-exposed worms ($p<0.01$), and slightly decreased it, however not significantly, after 28 days in both worm populations.

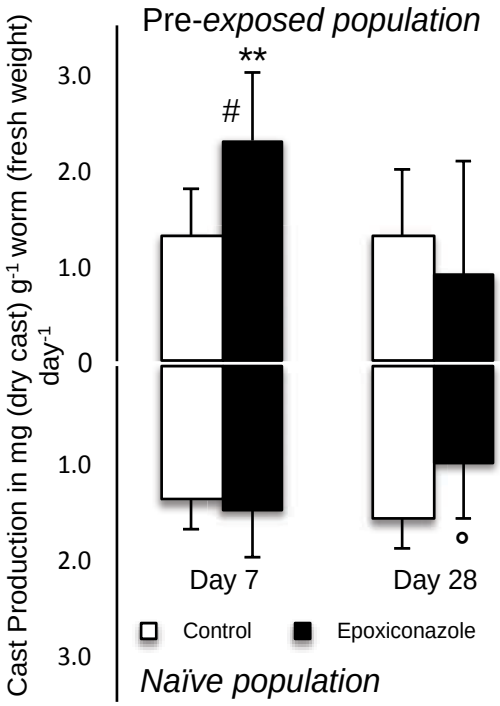

Fig2

Mean cast production (in g dry cast weight g^{-1} earthworm body mass day^{-1}) of pre-exposed and naïve *Aporrectodea caliginosa* after exposure to epoxiconazole for 7 and 28 days (n=8). Error bars are standard deviations (SD). * indicates significant differences between exposed and control groups (student-t-test, ° $p<0.1$, * $p<0.05$, ** $p<0.01$) and # between earthworm populations.

3.3. Energy resources

Mean glycogen content was decreased by nearly 20 mg in fungicide-treated compared to control groups after 7 days in the naïve earthworms, and after 28 days in the pre-exposed

earthworms as shown in (Figure 3-A). At the end of the exposure, the worms originating from the conventional-treated field had a slightly lower glycogen tissue level than the naïve ones.

The lipid tissue levels (Figure 3-B) did not show any significant differences between un-exposed and fungicide exposed earthworms or between earthworms originating from the conventional or the organic-treated field. Lipid levels were lower in all treated groups compared to their respective controls, however it did not achieve significance. Protein contents (Figure 3-C) decreased similarly in the 4 groups of worms during the first 7 days. They were then significantly increased by fungicide treatment in both exposed and naïve populations compared to their respective controls after 28 days, with pre-exposed worms having a final protein amount almost twice as high as naïve ones.

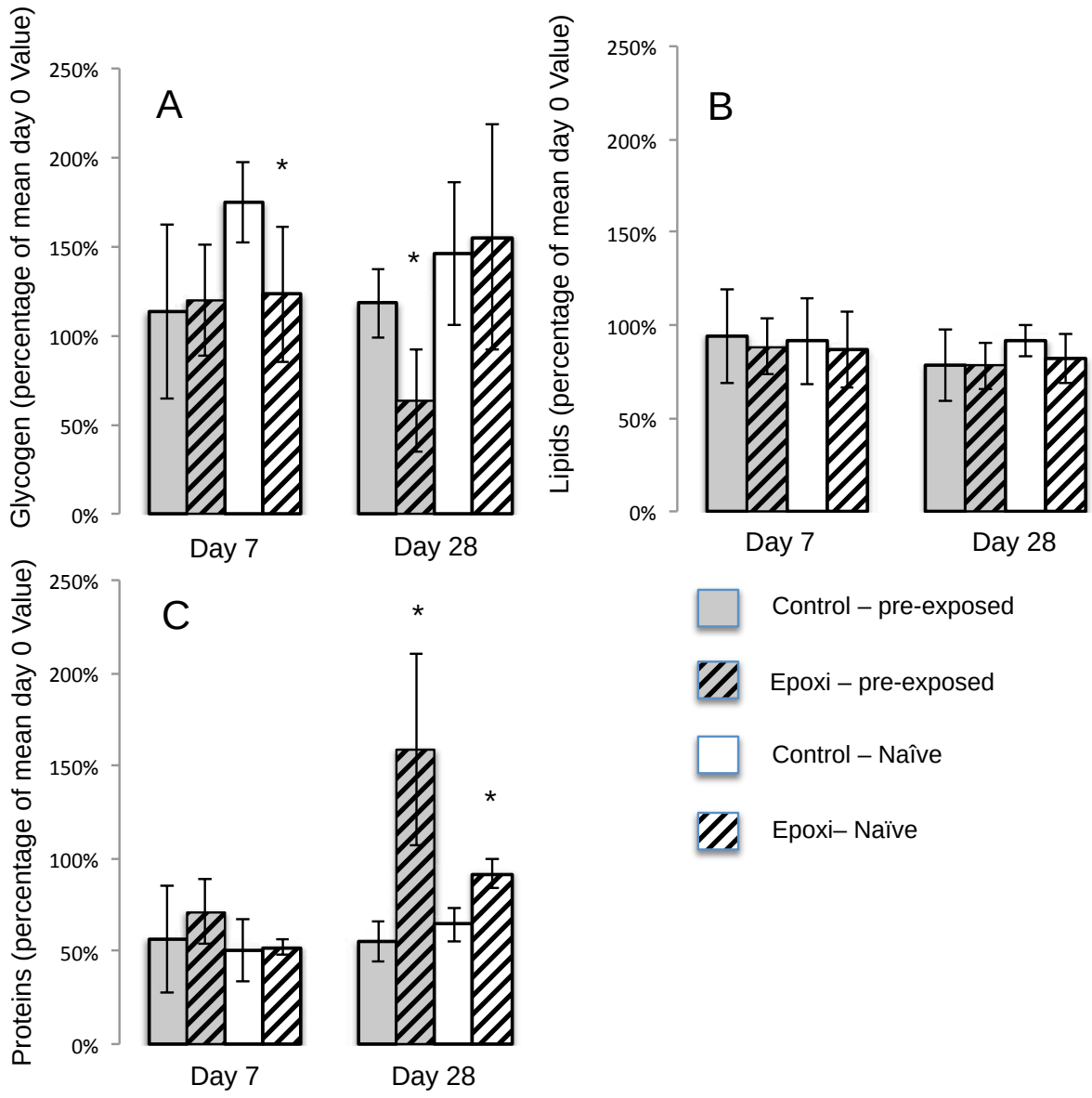

Fig3

Glycogen (A), Lipids (B), and Proteins (C) contents (percentage of mean of day 0 value) of pre-exposed and naïve *A. caliginosa* exposed to Epoxiconazole at day 7 and 28. Mean values (N=5 worm per group) are presented with standard deviations as *error bars*. Significant differences are indicated with * between exposed and control worms (Student-t-tests, $p<0.1$).

3.4. Metabolomics

3.4.1. Metabolic profiles of fungicide-exposed and control populations

Twenty eight metabolites were detected and quantified in the earthworm tissues. From this dataset, 22 were kept for interpretation (table 1). For the conventional population (Figure 4A), scores plots on axis 2 and 3 showed that the worms exposed for 28 days formed a separate cluster from the 28 days control worms along axis 2 and 3. This pattern was not observed in the organic population (Figure 4B) along any of the three axes, as it seems the time effect is greater than the fungicide effect. Indeed, the exposed and the control groups move in the same way with time, but are not clearly separated.

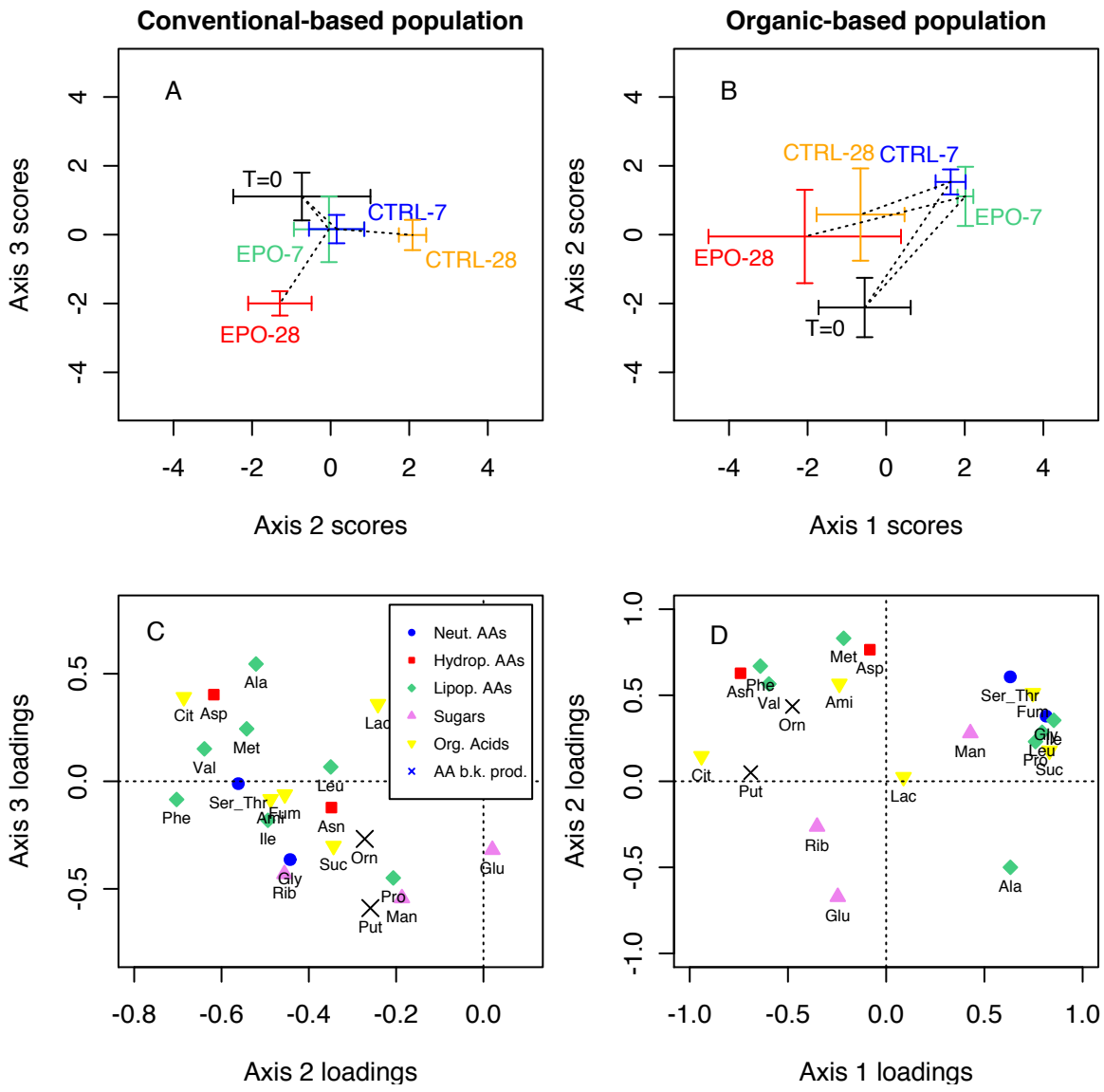

Fig4

Principal Component Analysis of metabolites data (22 variables) showing relationship between metabolite profiles and epoxiconazole exposure along time. A, B: Scores plots for conventional population (Axes 2 and 3) and organic population (Axes 1 and 2). Data are shown as crosses for both exposed and control groups means ± standard error of the mean (SEM). Exposed and Control groups are joined by time order with dashed lines. C, D: Loadings plots for individual metabolites in the conventional population (axes 2 and 3) and in the organic population (axes 1 and 2). Metabolites are identified by their abbreviations and colored by functional groups listed in supplementary material (Table S4).

3.4.2. Metabolic changes in the worms populations

In the loadings plots of the PCAs (figure 4C and 4D), several coordinated responses were identifiable. In the conventional population (Figure 4C), the 7 variables that have loadings lower than -0.5 on axis 2 form a first cluster containing mostly lipophilic amino-acids. Another cluster is in the lower half of the plot corresponding to variables having low loadings (roughly, <-0.2) on axis 3, composed of the three sugars, succinate, ornithine, the amino-acids glycine and proline and putrescine. On the other hand in the organic population (Figure 4D), we see two clear clusters that could correspond to the incubation time: the top right corner for seven days and the top left corner for 28 days. Day zero would correspond to the sugars Glu and Rib (lower half). The induced changes in metabolite concentrations after exposure was then measured as normalized concentrations in percentage of the control value and compared according to functional groups (Figure 5). In the conventional worms, several metabolites increased after 28 days of epoxiconazole exposure in all four biochemical groups (amino-acids, sugars, organic acids and ornithine-putrescine), up to 7-fold for aspartate, 4-fold for aminobutyrate, and 2-fold for ornithine and putrescine. This general increase in metabolite levels was not observed in the naïve population, which displayed different trends. Indeed, in Figure 5E, certain amino-acids (Asn, Asp, Phe and Val) increased slightly, whereas others dropped below 100% or stayed stable at 28 days. Not much variation was observed in organic acids of the organic earthworms at 28 days, except for aminobutyrate which dropped to 50% of the control value, contrasting to its 4-fold increase in the pre-exposed animals. Putrescine level increased by 2-fold after 28 days in both populations whereas ornithine decreased to below 50% of the control value in the organic population only.

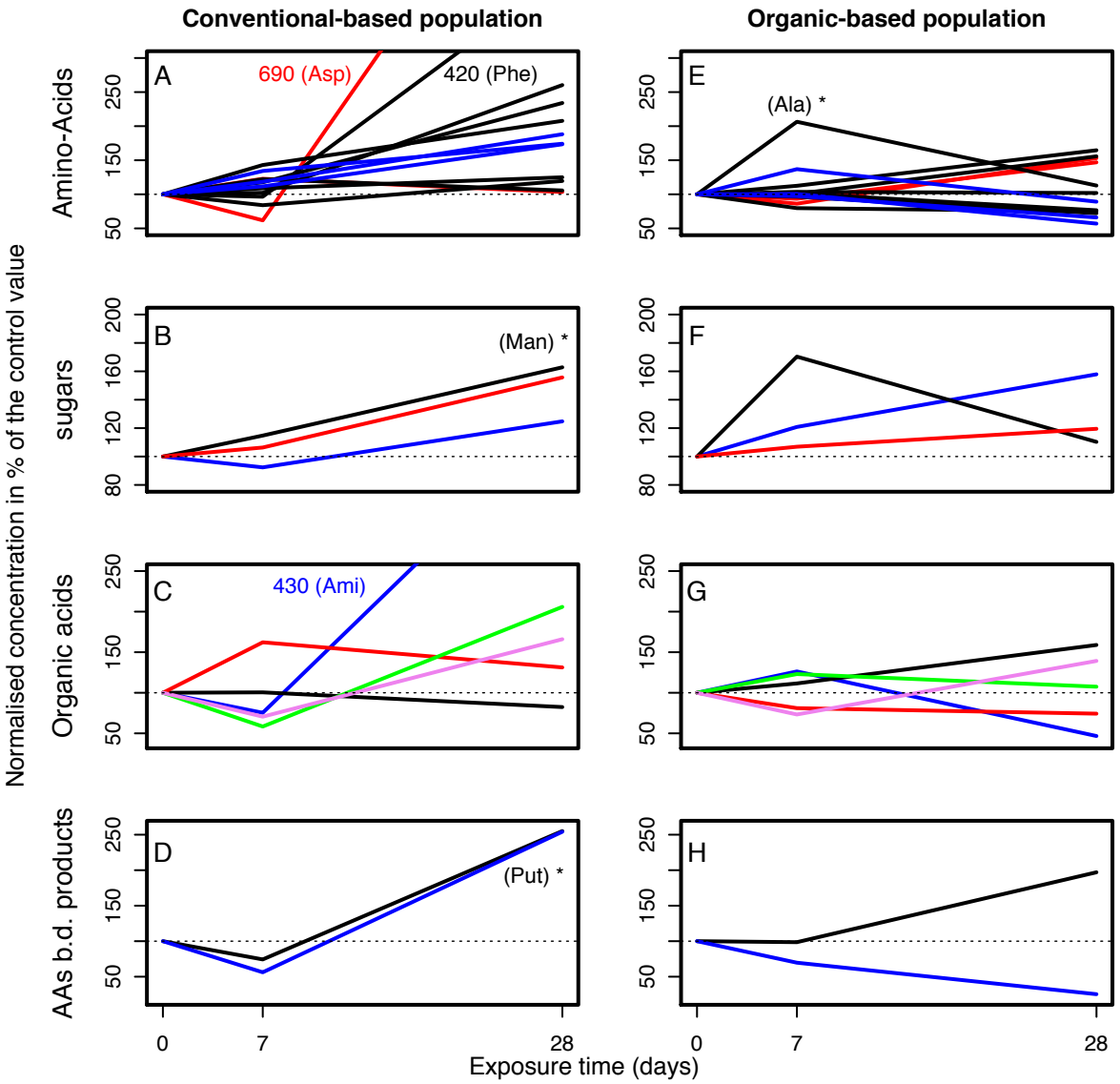

Fig5

Metabolite functional group responses to epoxiconazole, concentrations expressed as percentage of mean control value. A and E: amino-acids (blue=neutral, black=lipophilic, red=hydrophilic) B and F: sugars (blue=glucose, black=mannose, red=ribose) C and G: Krebs' cycle intermediates (blue = aminobutyrate, black = citrate, red = fumarate, green= lactate, violet= succinate) D and H: amino acids degradation products (black=ornithine, blue=putrescine). * indicates significant differences between exposed and control groups (Student t-tests, $p<0.1$).

4. Discussion

4.1. Acclimation in energetic processes and metabolism

Animals can tolerate pollutants via biotransformation, excretion or scavenging of free radicals (when pollutants induce oxidative stress). For example, detoxification of several pollutants, e.g atrazine or paraquat, is mediated via the enzyme Glutathione-S-Transferase. This enzyme, belonging to phase II detoxification mechanisms, acts through binding xenobiotics to glutathione and facilitating its excretion (Anderson and Gronwald, 1991; Wiegand et al., 2000, 2007). Another common detoxification pathway to xenobiotics is the group of cytochrome p450 oxidases. This family of enzymes transform the structure of organic chemicals, hence greatly altering their toxicity (Ribera et al., 2001; Rodríguez-Castellanos and Sanchez-Hernandez, 2007). However, no data on epoxiconazole tolerance pathways e.g. detoxification mechanisms was available in the literature.

On the other hand, the literature suggests that there are energetic costs in organisms for coping with pollutants (Wiegand et al., 2007; Fisker et al., 2011; Holmstrup et al., 2011). The way organisms handle energetic processes can therefore inform us on adaptation mechanisms. As an example by measuring the main energy resources, Pook et al (2009) showed that a metal-resistant population of marine harbour ragworm (*N. diversicolor)* had a lower scope for growth than a reference (non-resistant) population and demonstrated a metabolic cost, or tradeoff, of resistance. Other tradeoffs can be the co-selection of traits along with tolerance to xenobiotics, such as life history traits. Interestingly, in the conventionally cropped field, the worms sampled had a lower initial mean weight but with constant tissue composition compared to the organic worms (Table 1). This could be a result of the selection of smaller individuals, by the global agricultural management of the field, as a possible combined effect of fertilizers, pesticides and tillage (tillage was less frequent in the organic field due to the lucerne cropping). This assumption is supported by the fact that the bigger anecic species such as *L. terrestris* or *A. giardi* are usually the most impacted in cropped soil (Edwards and Bohlen, 1996).

To investigate such energetic costs, we measured the worm's metabolic rate (µg CO2 g^{-1} worm (fresh weight) $hour^{-1}$) as a proxy of energy dissipation, and the main energy storage compounds which are glycogen (main sugar resource), total proteins and lipids. Metabolic rate was increased in both populations after 7 days of exposure, showing that the fungicide increased metabolic rate, but to a greater extent in the pre-exposed earthworms. Energy dissipation in both control and exposed groups were indeed higher than the naïve earthworms after 28 days. The increase in putrescine and alanine observed in the two populations when exposed suggests a stress response in both groups of earthworms, these two metabolites have previously been suggested as universal biomarkers in metabolomics studies (Rhee et al., 2007; Groppa and Benavides, 2008; Simpson and McKelvie, 2009). However, the increase in most amino-acids contents in the pre-exposed earthworms after 28 days, which is not observed in the naïve animals, indicates a particular metabolic response. Several studies reported that the available resources in amino-acids were at the centre of metabolic activity during stress responses (Simpson and McKelvie, 2009; Krasensky and Jonak, 2012; Lankadurai et al., 2013). Moreover, the increase in alanine, aspartate, aminobutyrate and succinate observed in the pre-exposed populations could indicate an activation of the alanine, aspartate and glutamate pathway (Kanehisa and Goto, 2000).

The increase in both metabolic rates was reflected by depletion in the lipid and the glycogen resource. However a temporal delay appeared in the glycogen usage, indicating a differential mobilisation of this sugar resource between naïve and pre-exposed earthworms. The naïve earthworms seem to consume glycogen earlier than the pre-exposed group. Glycogen breakdown is reflected by the slight increase of glucose in fungicide-exposed groups of earthworms after 28 days. Soluble proteins were also significantly higher after 28 days in the pre-exposed worms only, which could indicate the higher synthesis of detoxification enzymes such as cytochrome p450 (Lukkari et al., 2004). Metabolic profiles of control and exposed groups in pre-exposed earthworms became distinct after 28 days, indicating that metabolic networks have been rearranged to maintain internal homeostasis and performance of the organisms.

Overall, all these findings show that pre-exposition of earthworms over generations in the conventional farming system has led to physiological adaptation, as evidenced by their higher reaction to the fungicide. Other studies have shown that separation of metabolic signatures (PCA analyses) increase with higher doses of the pollutants, e.g DDT, endosulfan or copper

(Bundy et al., 2008; Simpson and McKelvie, 2009). It is likely that, in our study, fungicide recommended application rate corresponds to a low sublethal dose and the differences in metabolic signatures would become clearer with higher concentrations of the pesticide. Nevertheless, the differences observed demonstrate an impact even at this environmentally realistic level. As both populations were selected from fields under long term (20 to 25 years) conventional and organic farming, an adaptation mechanism on the genetic level could be assumed, but would need to be proven.

4.2. The link with earthworm burrowing and the pesticide fate in soil

Several studies aimed to correlate biochemical or cellular responses in earthworms to pollutants with ecologically important endpoints. For example, Maboeta et al. (2001) showed that there was a link between decrease in the abundance of field populations of the earthworm *Microchaetus sp*. and decrease in the animals' neutral red retention time (a biomarker of cellular damage). By showing a strong reduction of earthworm growth by pesticides, (1992) postulated that these contaminants were likely to cause a delay in sexual maturity in juveniles and have eventually have an impact on earthworm abundance in the field. However, pesticide impacts on burrowing behavior have only poorly been studied because of the difficulty to visualize or estimate burrowing activity, and few studies have tried to link pollutant impacts at low levels of organisation (cellular, biochemical) with earthworm burrowing (Capowiez et al., 2010; Gupta and Sundararaman, 1991). With regard to the ecological importance of earthworms through the burrowing of the soil, it is likely that, when attempting to assess the ecosystem services rendered by earthworms to the soil, earthworm burrowing behaviour is as important as population numbers as it can have drastic impacts for soil functioning (Capowiez & Bérard 2006).

Here, the low dose application of fungicide resulted in an increase in cast production after seven days, which is consistent with the recent results of Dittbrenner et al (2010), where cast production was increased only at the lowest concentration of the pesticide, but decreased at higher doses. The impacts of pesticides on soil bioturbation have been investigated in a few articles using 2D and 3D (X-ray tomography) terraria, cast production method and the avoidance behaviour test. Most of them, except for the paper of Dittbrenner et al (2010), have shown a decrease in activity or an impact on the characteristics of the burrow systems, e.g

length, depth, and branching rate (Pelosi et al., 2013). Interestingly, in the present study, the increase in cast production was only observed in the pre-exposed earthworms. Therefore it could suggest that tolerance to this environmentally realistic level of fungicide is associated with a compensatory increased activity. This increase in burrowing behaviour could be induced by the metabolic changes observed in energetic depletion and metabolites rearrangements, similarly to the phenomenon of hormesis (Zhang et al., 2009). An alternative hypothesis would be that it is related to avoidance behaviour, but unsuccessful, as the earthworm is confined to the microcosm, resulting in an increased amount of casts. It is also known that geophagous earthworms are able to alter their burrowing behaviour and display different behaviours (in terms of soil ingestion) when they are feeding on organic matter in the soil or moving through the soil (possibly as part of an avoidance response) (Capowiez and Bérard, 2006; Hugnes et al., 1996). This may lead to reduced cast production in contaminated soils rather than increased cast production but it is dependent on the level of contamination and the contaminant (Dittbrenner et al., 2010).

The fate of pesticides in soil can be affected by earthworms bioturbation via several mechanisms. It increases pesticides sorption on soil particles on the long-term, leading to the formations of non-extractable residues. Therefore it can increase the pesticide persistence, as it was previously shown for atrazine (Binet et al., 2006; Farenhorst et al., 2000). On the other hand, earthworms' activity was also reported to stimulate microorganisms activity, and enhance the activity of atrazine- or MCPA-specific bacterial degraders, accelerating its mineralisation (Liu et al., 2011; 2011, 2008). *A. caliginosa* also participated in the breakdown of four fungicides (folpet, fosetyl-Al, metalaxyl, myclobutanil) and two insecticides (Chlorpyrifos-Ethyl and λ-Cyhalothrin) (Schreck et al., 2008). In our study, pesticide concentration is lower in the microcosms containing earthworms from the conventional field (Table 2). The increase in bioturbation observed in these earthworm's microcosms suggest that they play a part in the pesticide's disappearance either by enhancing sorption or by enhancing microbial mineralization of epoxiconazole.

5. Conclusion

This study shows that an environmentally realistic concentration of epoxiconazole applied as OPUS® induced distinct physiological changes in two populations of the earthworm *A.*

caliginosa. Biological responses in energy storage and metabolic profiles differed between earthworms derived from conventional farming and those from organic farming, indicating that an acclimation mechanism to the agricultural pesticide occurs in the long-term pre-exposed animals. The acclimation in pre-exposed animals was also evidenced by their higher reaction to the chemical, with increased metabolic rate and burrowing activity compared to the naïve animals, which ecological consequence is a lower pesticide concentration in the soil. This original finding is of major interest in the frame of ecosystem resilience to global changes. Whether this physiological adaptation is a general pattern of response against fungicides or other pesticides would need to be confirmed with other molecules and agricultural contexts.

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Supplementary materials

Table A

Comparison of the initial earthworm weights (n=85) collected from the conventional and organically treated fields for use in the container experiment. Five earthworms were selected from each group and analysed for total glycogen, soluble proteins, lipids and basal respiration rate measured. Results are expressed as the mean ± standard deviation. Significant differences between the two populations are indicated by different letters in the same column (Student t-tests, $p<0.05$).

Worms from field	Initial weight g FW		Glycogen mg g^{-1} dry worm		Proteins mg g^{-1} dry worm		Lipids mg g^{-1} dry worm		CO_2 µg CO2 g-1 FW d-1	
Organic	0.60	$\pm 0.14_a$	36.6	$\pm 17.5_a$	96.6	$\pm 20.1_a$	169.6	$\pm 20.3_a$	44.7	$\pm 32.3_a$
Conventionnal	0.28	$\pm 0.04_b$	33.0	$\pm 24.8_a$	89.9	$\pm 21.6_a$	178.6	$\pm 31.9_a$	62.2	$\pm 35.2_a$

Table B

Fungicide contents in soil used for the experiment, and in treated soil with and without earthworms at day 0, 7 and 28. Measurements were realised in triplicates (extraction and analyse) from the same sample of soil. Results are expressed as mean ± standard deviation.

Soil	Epoxiconazole levels (ng.g^{-1} dry soil)		
	Day 0	Day 7	Day 28
Un-treated (control)	nd	-	-
Fungicide-treated without earthworms	79.8_a ± 6.9	62.7_a ± 12.6	57.8_a ± 8.3
Fungicide-treated with naïve *A.caliginosa*		70.1_a ± 23.5	58.6_a ± 14.8
Fungicide-treated with pre-exposed *A.caliginosa*		57.8_b ± 5.3	52.6_b ± 8.6

Significant decrease in pesticide concentration from the initial day 0 value (One way ANOVA with time as factor followed by Tukey post-hoc tests, $p<0.05$).

nd = not detected

Table C

Weight change of pre-exposed and naïve earthworms at days 7 and 28. Values are mean out of 5 replicates (N=5) ± *standard deviation* (SD) per group. No significant effect of time on earthworm weight throughout the study was observed (one way anova with time as factor).

		Weight change after exposure in % initial weight	
Population	Soil treatment	Day 7	Day 28
Pre-exposed	Epoxiconazole	98.3% ± 13.1%	111.4% ± 24.7%
	Control	95.2% ± 20.6%	81.0% ± 12.5%
Naïve	Epoxiconazole	98.2% ± 25.3%	85.8% ± 11.4%
	Control	92.8% ± 5.3%	86.6% ± 12.9%

Table D:

List of metabolites identified in freeze-dried tissues of the earthworm *Aporrectodea caliginosa.* Metabolites kept for interpretation were classified into four functional metabolic groups: amino-acids, sugars, organic acids and urea cycle metabolites from the energetic metabolism. The others metabolites found but not kept for interpretation are classified as polyols, other metabolites, and non quantified metabolites (below the quantification limit of the GC-MS). For the quantified metabolites, minimum and maximum concentrations (nmoles/mg dry mass) are also shown.

Metabolites	Abbreviation	**Minimum and maximum concentrations (nmoles/mg dry mass)**
	Amino acids (12)	
Hydrophilic Amino-Acids (2)		
Asparagine	Asn	0.34-8.86
Aspartate	Asp	0.16-2.65
Lipophilic Amino-Acids (7)		
Alanine	Ala	0.02-17.1
Isoleucine	Ile	0.34-0.41
Leucine	Leu	0.03-0.61
Methionine	Met	0.32-0.62
Phenylalanine	Phe	0.02-0.69
Proline	Pro	0.35-0.61
Valine	Val	0.13-1.54
Neutral Amino-Acids (3)		
Glycine	Gly	0.15-8.49
Serine	Ser	0.16-2.24
Threonine	Thr	0.18-0.67
	Sugars (3)	
Glucose	Glu	0.15-0.96
Mannose	Man	0.02-0.14
Ribose	Rib	0.02-0.2
	Organic acids from the energetic metabolism (5)	
Aminobutyrate	Ami	0.03-0.84
Citrate	Cit	0.02-0.58
Fumarate	Fum	0.16-3.68
Lactate	Lac	0.03-20.84
Succinate	Suc	0.16-5.33
	Urea cycle metabolites (2)	
Ornithine	Orn	0.02-0.92
Putrescine	Put	0.21-3.78
Metabolites not included in the interpretation		

Polyols (4)	
Glycerol	0.62-13.81
Glycerol 3 phosphate	0.16-8.04
Inositol	0.41-4.05
Mannitol	0.02-2.46
Other metabolites (2)	
Cytosine	0.02-0.26
Ethanolamine	0.02-0.41
Non quantified metabolites (9)	
Glycerate	
Pipecolate	
Erythritol	
Citrulline	
Fructose	(< QL , S/N < 10)
Galactose	
Sorbitol	
Gluconate δ-lactone	
Trehalose	

Manuscript 3:

LIFE TRAITS OF COCOONS AND RESPONSE TO ORGANIC SOIL CONTAMINATION IN EARTHWORM POPULATIONS FACING DIFFERENT AGRICULTURAL PRACTICES

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In preparation

Life traits of cocoons and response to organic soil contamination in earthworm populations facing different agricultural practices

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Abstract

Earthworm populations have to face agricultural management in cropped fields, in particular pesticide uses. We asked whether cocoons traits respond to agricultural management and what insight into their impacts they can provide. Several endpoints of life history were compared in F1 earthworms *Aporrectodea caliginosa* bred from populations originating from a conventional field (i.e. pesticide-exposed population) or an organic field (i.e. reference population), allowed to reproduce in clean soil. On the cocoons obtained from these adults (F1 generation), initial cocoon and juvenile weights, cocoon wall thickness, and cocoon hatching times and rates were measured. Juvenile growth of F1 was followed for 90 days in clean control soil and in contaminated soil by two pesticides (glyphosate and epoxiconazole applied individually and as a cocktail) frequently applied on the conventional field.

The "pre-exposed"population from the conventional field revealed significantly lower adult and cocoon weights, longer hatching times and lower hatching rates but no differences in cocoon wall thickness, compared to the reference unexposed population from the organic field. Juveniles of the two populations exhibited similar mean hatching-weight (11,0 + 3.3 and 12,2 + 3.0 mg f.w. for naïve and pre-exposed populations, respectively). Growth of the juveniles was not markedly different between the naïve and pre-exposed populations, the ones originating from naïve adults growing slightly faster, although significance failed due to high individual variation. However juveniles growth was visibly reduced (around 50%) in juveniles of the exposed population, when growing in glyphosate contaminated soil.

Agricultural management, and in particular the use of pesticides, decreases the mean adult weight in our experimental fields, which probably leads to smaller cocoons, and lower hatching success. Such changes in life traits of cocoons and in ability of juveniles to cope with soil contamination might have adverse consequences on the population dynamics and might also explain reduced densities of earthworms in such ecosystems.

Keywords

Life history traits, *Aporrectodea caliginosa,* cocoons, growth, pesticides, soil organic contamination

1. Introduction

While achieving increasing productivity, agricultural practices are nowadays greatly affecting soil biodiversity and the ecosystem services it sustains. Because the sustainability and fertility of the soils depend on their biological component, there is a need to quantify the role of organisms such as earthworms in soil processes. Indeed, the ecosystem services they sustain (e.g soil formation, water regulation or nutrient turnover) are meant to be replaced by human inputs such as harrowing, irrigation, chemical fertilization or pesticide applications (Giller et al. 1997; Barrios 2007; Blouin et al. 2013) (Paoletti 1999; Barrios 2007; Jouquet et al. 2007).

Earthworms represent the largest soil biomass and are considered as ecosystem engineers, however their communities are drastically affected by agricultural practices. Pesticides in particular can have detrimental impacts on life history traits of earthworm populations, including reproduction, survival or growth, which are particularly relevant for evaluating effects at population level (Neuhauser & Callahan 1990; Springett & Gray 1992; Booth & O'Halloran 2001; Spurgeon et al. 2004; Bindesbøl et al. 2007).

Despite the known effects of many pesticides on life-cycle parameters, earthworm populations persist in pesticide-contaminated soils, albeit in reduced numbers and diversity (Smith et al. 2008; Pelosi et al. 2013). This strongly suggests that the long-term exposure of earthworms to residual pesticides in agricultural fields led to selection of pesticide-adapted populations. Adaptation to pollutants can take place through several mechanisms, e.g. avoidance behaviour, physiological resistance by increased detoxification or sequestration of the pollutants (Posthuma & Van Straalen 1993) but also through selection of life history characteristics (Spurgeon & Hopkin 1999). Metal-resistant populations isolated from metal-contaminated areas of the terrestrial isopods *Porcellio scaber* expressed early reproduction and increased reproductive allocation (Donker et al. 1993). Metal tolerance mechanisms and occurrence in invertebrates is well described (Posthuma and Van Straalen, 1993). However to the extent of our knowledge investigations of adaptation to soil organic contamination by pesticides in agricultural fields in non-target organisms such as earthworms are still scarce.

This study tests the hypothesis that long-term exposed earthworms inhabiting soil of a conventional cropped field have developed adaptive responses to pesticides in terms of life cycle parameters. Life cycle parameters were compared in two independant populations of the

earthworm *Aporrectodea caliginosa* isolated from either a conventional cropped field (with pesticides applications) or an organic cropped field (without any pesticide applications), both in this management for more than 20 years. Population differences were then investigated as adult, cocoon and juvenile weights, cocoon wall thicknesses, cocoon hatching times and rates, and growth of juveniles when experimentally exposed to two pesticides (individually and as a cocktail) frequently applied on the conventional field.

2. Materials and methods

2.1. Origin of selected populations and rearing of animals

Earthworms used in the study originated from two agricultural fields located in the same agricultural area (Vézin-le-Coquet, Bretagne, France). One was conventional cropped (mainly cereals-maize-protein crops rotations) for more than 20 years with roughly 6 pesticide applications per year, in addition to 1-2 times organic (pig manure) or chemical fertilization per year, and mechanical treatment using both mechanical and chemical weeding. The other one was a field cropped according to organic agriculture requirements since 1992 (mainly cereal-proteaginous rotations), applying no chemical pesticides and only organic fertilizers, but performing mechanical weeding. Soils are slightly acid silt-clay loams (conventional and organic field, respectively: Clay 14.8 % and 16.6 %; Silt 71.6 % and 71%; Sand 13.6 % and 12.4 %; organic matter 1.67 % and 2.55 %; pH_{water} 6.4 and 6.9; calcium content 0.36 % and 0.30 %). The endogeic species *Aporrectodea caliginosa* was choosen as commonly found and representative earthworm population in agricultural landscapes. Adults *Aporrectodea caliginosa* (presence of a fully developed clitellum) were recovered during autumn 2011 by hand sorting from the two fields. They were brought back to the laboratory in humid soil from their home fields, rinsed in tap water, gently dried on filter paper and weighed. Soil used for the exposure experiment was collected from the first 30cm of a permanent (since 1960) organic pasture (17.6% clay, 69.3 % silt, 13.1% sand, 4.0 % organic matter, pH 6.0, calcium content 0.3 %) located in the same area. Pasture soil was passed through a 4mm sieve and supplemented with 2% of a mixture of dry grass and horse manure (1:1). Culturing vessels consisted of 30 cm-diameter buckets sealed with a lid pierced with tiny holes to ensure sufficient aeration and containing 2.5 kg (dry weight) of this supplemented soil re-moistened

at 25% H_20 and kept in a climatic room (ERATIS; temperature: 15°C; day/night cycle: 16/8h; humidity: 80 ± 5%). 15 adult earthworms were placed in five culturing vessels per earthworm population (75 per population). Once per week, earthworms were removed from the culturing vessels and soil was manually sorted for cocoons. Cocoons were rinsed in tap water, gently dried on filter paper and weighed. They were then placed in 24 well plates in distilled water and kept at 4°C until enough cocoons were obtained (Lowe & Butt 2005). All weight measurements of cocoons and juveniles were made with a 0.001 mg precision Sartorius Micro M2P balance (Sartorius, Aubagne, France).

2.2. Hatching procedure

After sufficient cocoons were obtained to make a large enough cohort from the culturing vessels, cocoons were placed individually in a 24 well plate to monitor hatching times and rates. Each well of the plates contained 1.5 g of pasture soil sieved at 1mm and moistened to 50 % H_20, covered with a moist filter paper to maximize humidity level. They were then incubated in another climatic room (CONVIRON GR96, 20°C, day/night cycle: 16/8h; humidity: 80 ± 5%). Each day the plates were examined for hatched cocoons, juveniles were weighed at hatching and kept in the same soil from the growth exposure at 12°C to slow their development until the start of the exposure. Juvenile viability was calculated as the number of viable juveniles divided by the number of hatched cocoons.

2.3. Juvenile growth experiments under pesticide exposure

The two most quantitative and frequently applied active pesticide molecules in the history of the conventional field were the fungicide epoxiconazole and the herbicide glyphosate (Givaudan et al, to be published). Hence these two molecules were chosen for the experimental exposure. The same pasture soil was used for the experiment in the microcosms. Upon retrieval, it was air-dried before being sieved at 2mm and kept in closed bins until used for the experiment. Both pesticides (alone or in combination) were applied as commercial formulations OPUS® (125 g active ingredient l^{-1}, obtained from BayerCropScience) and RoundUp Flash® (450 g active ingredient l^{-1}, obtained from Monsanto). Opus® and RoundUp® were applied at predicted soil concentrations of the active ingredient of 0.1 $\mu g.g^{-1}$ dry soil and 2.5 $\mu g.g^{-1}$ dry soil, calculated for field application rates of 1 $l.ha^{-1}$ and 4 $l.ha^{-1}$,

respectively. Concentrations were calculated assuming a single application with an homogenous distribution, a soil density of 1.5 $kg.l^{-1}$, and no crop interception in the top 5 cm of the soil (Dittbrenner et al. 2010). Soil spiking was conducted by manually adding diluted pesticide solution or distilled water (for the controls) on soil at 10% water content, then adding extra distilled water in order to reach a final humidity of 30% H_20. To insure homogeneity of pesticide distribution in the soil, the solution was added in two steps with the soil being thoroughly mixed, redisposed as a fine layer and resieved at 2mm. Soil microcosms consisted of polycarbonate boxes (50mm diameter x 30mm height, Caubère, Yebles, France) that were filled with 30 g of contaminated or control soil spiked with pesticides and/or distilled water, respectively and left two days in a cool dark room to ensure aeration of the soil after re-humidification. The soil (with or without pesticide) was renewed every two weeks. A single juvenile was introduced per soil microcosm. The experimental design comprised 8 exposure treatments (2 populations exposed to epoxiconazole, glyphosate and their mixture plus non exposed controls) of 5 replicates each.

In order to follow the juvenile development, each juvenile earthworm was rinsed in tap water, gently dried on filter paper and weighed twice on the first month, then weekly from days 50 to 90.

2.4. Cocoon phenotype: morphological and histologic measurements

30 cocoons from each population (about 1 week old) were fixed in paraformaldehyde 4% for one day, immersed in Phosphate Buffer Saline (PBS) 0.1% then placed in 30% sucrose in PBS. The cocoons were then embedded oriented apex up in $1cm^3$ "NEG-50" frozen section medium (Microm Microtech, Francheville, France). 40 µm frozen sections were made with a cryostat-microtome apparatus (Cryo-Star HM 560M, Microm Microtech, Francheville, France) at -30°C, and mounted on slides. The slides were stained by hemalun eosin safran (HES) and digitalized by the nanozoomer 2.0HT (Hamamatsu, Japan) on the histopathology core facility H2P2 (University of Rennes1) in order to highlight the cocoon walls. One section containing the apex (hence in the mid-sagittal plane) was kept for each cocoon chorion thickness measurement. Approximately 40 measurements (1 measurement every 0.2 mm) were realised on each cocoon to cover the entire wall with NDP.view software (Hamamatsu

Photonics, Massy, France) as shown in figure 1. The mean of all values from each cocoon was computed and used for statistical analyses.

2.5. Statistical analyses

Differences in cocoon weights were tested by an Analysis of Covariance (ANCOVA) with cocoon weight as dependent variable, juvenile weight as independent covariable, and population as categorical factor. The weight of the chorion (i.e. “empty” cocoon) was computed by substracting the juvenile weight at hatching from the whole cocoon weight. Differences in juvenile weights, chorion weights, hatching times and cocoon chorion thicknesses between the two populations were tested by student-t-tests. Differences of adult mean weights between populations or between control and pesticide-exposed juveniles during the growth experiments were tested by student-t-tests. Hatching percentage and percentage of viability of the juveniles were tested by Chi-square tests. All statistical analyses were realised with R version 2.12.1 (R Development Core Team 2008).

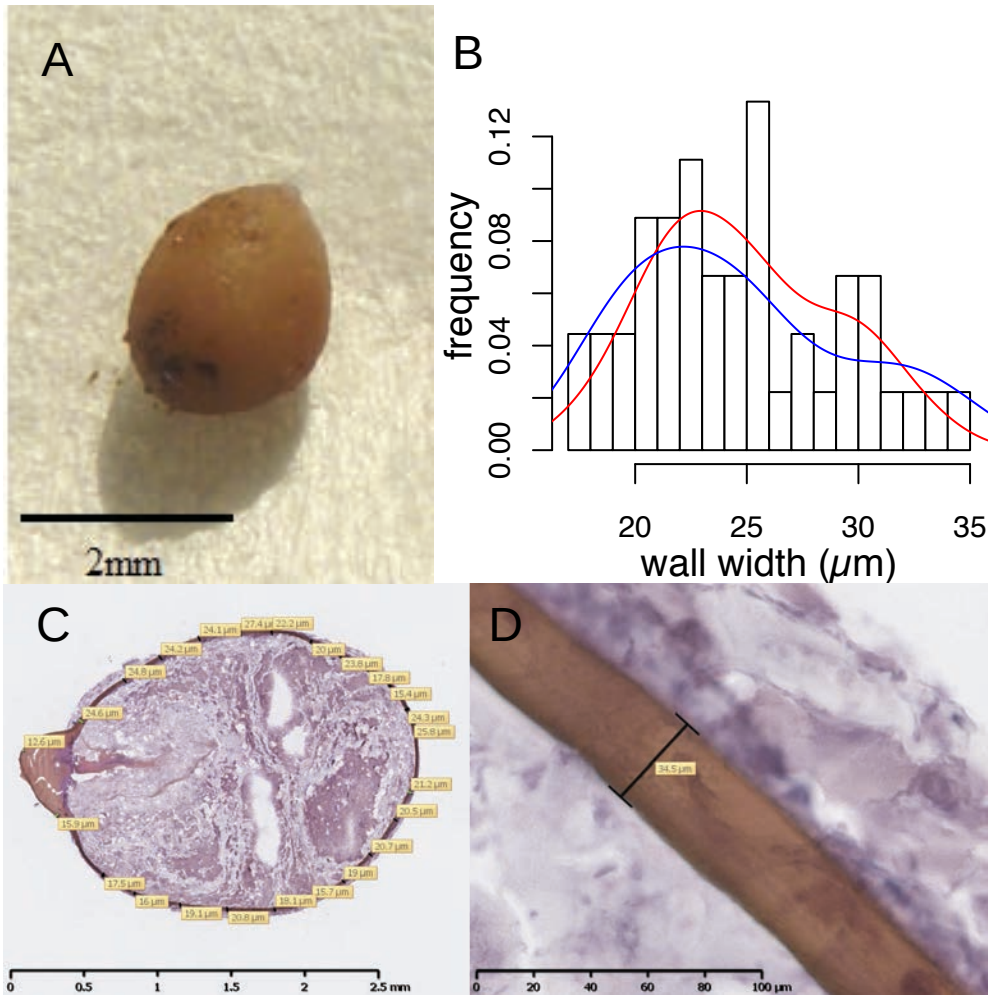

Figure 1:Coocon phenotype of *Aporrectodea caliginosa* . A: whole A. caliginosa cocoon picture, B: frequency distribution of cocoon chorions thickness in both populations. The red and blue lines are the Kernel density distributions of cocoon chorion thickness in the conventional field population and the reference field population, respectively. C: whole cocoon section picture after HES coloration and measurements along the cocoon wall. D: 40x zoom picture of a single cocoon chorion measurement

3. Results

3.1 Long-term adaptation of life traits

The total number of cocoons obtained was 74 for the pre-exposed population and 185 for the reference population. Cocoon production was very variable between vessels and ranged from 0.13 to 0.77, and from 0.37 to 2.53 cocoons.adult^{-1}.week^{-1} for the conventional field and the organic field population, respectively. Mean weights of adult earthworms sampled from the conventional field were markedly lower than earthworms from the organic field (table 1).

Mean cocoon weight was also lower in the conventional population compared to the organic one ($p<0.05$).

A close correlation between juvenile and cocoon weights was verified (ANCOVA, $p<0.001$, adjusted $R^2=0.45$). When statistically controlling the variance due to juvenile weight through the Analysis of Covariance (ANCOVA), mean cocoon weight was significantly higher in the reference population ($p<0.001$ for the factor "population"), although cocoon chorion thickness (25 µm) was unchanged between the two populations (Table 1). Interestingly, cocoon chorion weight at hatching was also significantly higher in the naïve population ($p < 0.001$). Hatching time was significantly longer and hatching rate lower, respectively, in the conventional field compared to the organic field while juvenile viability (48%) was similar in both populations

Table 1: Life traits of adult *A. caliginosa* earthworms (N=150) and their offspring sampled on a conventional and an organic cropped field. Mean weights of cocoons and juveniles produced per population of origin and mean differences between cocoon weights and juvenile weights (N=109); mean hatching times and rates, and juvenile viability (N=109); mean cocoon wall thickness (N=47). Significant differences are indicated in bold

		Population and field of origin	
		pre-exposed conventional	naïve organic
Weight	Adults	417 ± 171 mg	**662 ± 193** mg
	Cocoons	12.50 ± 2.30 mg	**13.72 ± 6.30** mg
	Juveniles at hatching	12.24 ± 3.32 mg	11.04 ± 3.39 mg
	Cocoon chorion	0.54 ± 0.38 mg	**2.39 ± 0.31** mg
Hatching	Hatching time	30.71 ± 3.96 days	**28.34 ± 5.10** days
	Hatching rate	64 %	**81** %
	Juvenile viability	48 %	48 %
Cocoon chorion	Thickness	24.82 ± 4.02 µm	24.61 ± 4.98 µm

3.2 Juvenile growth as affected by soil pesticide contamination

No mortality occurred during the 90 days of the growth experiments, except one worm in the epoxiconazole-treated group (in the conventional field population) and one worm in the glyphosate-treated group (in the organic population),. In uncontaminated control soil, the juveniles from both populations grew without marked differences, the ones originating from the reference population grew slightly faster. However, glyphosate soil contamination modified juvenile growth rates, with the juveniles from the reference population growing faster than the ones from the conventional field population (figure 2A, although it failed significance tests due to high individual variations.

In particular, glyphosate soil contamination resulted in around 50% lower mean weight of juveniles of pre-exposed earthworms from day 29 onwards for the entire exposure duration.

Juveniles from naïve earthworms were affected by glyphosate in the beginning, but recovered to control values between day 29 and 53 and onwards. A different trend was showed with the fungicide soil contamination (Fig. 2) with expoxiconazole rapidly lowering the growth of juveniles from organic earthworms but only for the first 40 days (2B), then the growth rates merged and remained close to the control value. The apparent increase of growth rate of juveniles of pre-exposed population under epoxiconazole soil contamination seems to be a bias due to the fact that juveniles from the pre-exposed population grew slower in the controls. Relative growth rates (fig 2B) seemed to be highly influenced by epoxiconazole for the first 40 days. The fungicide seemed to increase the growth of juveniles from conventional earthworms, and decrease the growth of juveniles from organic earthworms, then the growth rates merged and remained close to the control value. This however, is due to a difference in the controls, as juveniles from the pre-exposed population grew slower. Glyphosate treatment resulted in around 50% lower mean weight of juveniles from pre-exposed earthworms from day 29 onwards for the entire exposure duration. Juveniles from naïve earthworms were affected in the beginning, but recovered to control values between day 29 and 53 and onwards. The pesticide mixture seemed to have no effect on juveniles from the conventional population, whereas mean weights of organic population were lower than the control values. Due to the high individual variation between growth rates, none of these trends achieved statistical significance.

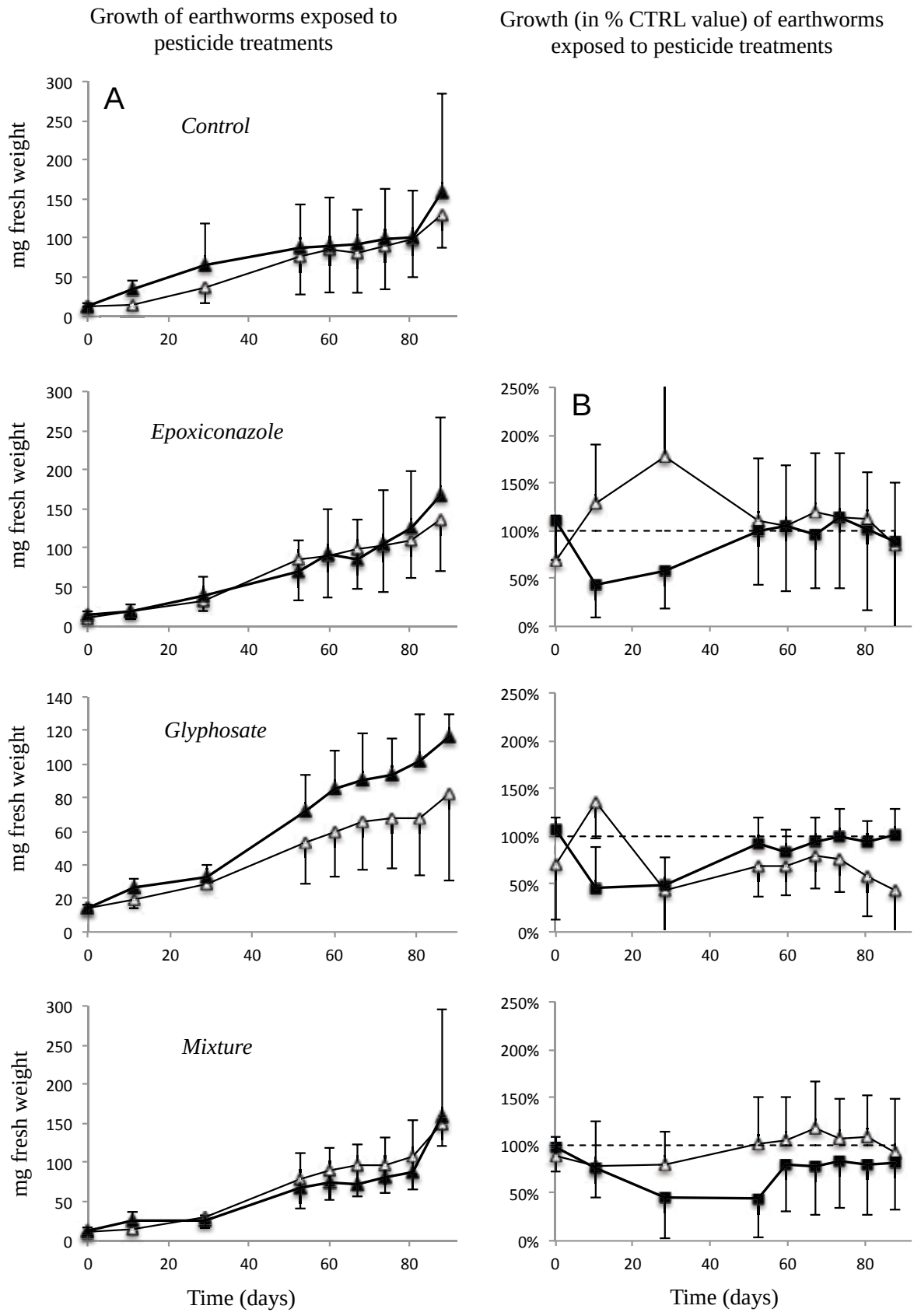
Growth of earthworms exposed to pesticide treatments
Growth (in % CTRL value) of earthworms exposed to pesticide treatments
A
B
Control
Epoxiconazole
Glyphosate
Mixture
mg fresh weight
Time (days)
Time (days)

Figure 2: A: absolute and B: relative growth of earthworms (in percentage of the mean weight of the respective control group from the same population) during 90 days in pesticide-contaminated soil (epoxiconazole, glyphosate and their mixture). Mean weights ± standard deviation out of 4-5 replicates. Open symbols: conventional population, black symbols: organic population

Discussion

In this study, the two populations of A. caliginosa originating from different agricultural backgrounds differed in terms of adult and cocoon traits and in terms of response to pesticide soil contamination. Production of cocoons was markedly (but not significantly) reduced by a factor of 2.5 in the worms originating from the conventional field, despite they were sampled before the annual pesticide application and reared for the reproduction in non-contaminated soils. Exposure to endosulfan (insecticide) and fenamiphos (nematicide) at field application rates and methiocarb (molluscicide) at 10x the field application rate inhibited cocoon production in *Aporrectodea trapezoides* already after 48h (Choo & Baker 1998). In that study worms were exposed on moist filter paper, which delivers the toxic pesticides much faster than an exposure through soil contamination. Similarly, cocoon production and hatching success was reduced by exposure of juvenile and adult *A. caliginosa* to organophosphate insecticides chlorpyrifos and diazinon (Booth et al. 2000). Our study comparing response of naïve and long-term exposed population to realistic soil contamination, adds to the knowledge that long term exposure leads to an adaptation reaction in terms of reduced fecundity, (as evidenced here by lower hatching rates, even when the worms are not exposed. This adaptative finding is of importance as in turn reduced fecundity influence population density in conventional managed fields.

Despite the initial cocoon weight was significantly higher in the naïve population, the hatched juveniles did not differ in their hatching-weight between populations. Earthworm cocoons contain an albuminous fluid that serves as feeding resource for the developing embryos (Lavelle & Spain 2001). As the cocoon chorion width was the same in both populations, it is

likely that the cocoons from the naïve population contained more nutritive material than the ones from the pre-exposed population, which was left unused upon hatching. West *et al* (2003) suggested that populations of the epigeic earthworm L*umbricus rubellus* stressed by soils with low calcium content allocate less energetic resources to reproduction, thus having smaller cocoons. In this study the differences cannot be due to soil properties because they originate from the same agricultural area, although there is a small difference in organic matter content. Hence the differences observed are likely to be due to the agricultural management, and particularly the use of pesticides. It is possible that adult worms from the organic field are capable of depositing higher amounts of albuminous fluid, hence more energetic resources in the cocoon. Pesticide stressed adults may not be able to allocate as much energy to the cocoon as worms from a non-polluted environment because they have to allocate energy for detoxification (Givaudan et al, to be published). The lower amount of resources available in the cocoons from the pre-exposed population could then explain their two hatchability (longer time and lower success). Svendsen and coworkers (2005) reported an opposite pattern in the earthworm *Lumbricus terrestris*, as adults worms treated with the antiparasitic drug ivermectin produced cocoons with lower hatching times than control worms.

Cocoon sizes are known to be correlated with adult weights in several species of earthworms (Reinecke & Venter 1987; Jiménez & Thomas 2001). The mechanism behind this correlation is not clear, but it is believed that the bigger the clitellum of the adults is, the heavier the cocoon is. It is then possible that these differences in cocoon traits are linked to the difference of the parents weight. Adult weights in this study were higher in the naïve population, which is in accordance with our previous results from the same population one year later (Givaudan *et al*, to be published). Hence, part of the observed difference in the cocoon size may also be attributed to this fact. Nevertheless, the agricultural management and the use of pesticides apparently impacts the mean age and weight of the worms' population, hence having indirectly an impact on the mean cocoon weight and their energy content.

This could then have consequences for the population fitness in terms of cocoon hatching and juvenile viability. This would be an example of a cost of tolerance of pesticides in earthworms

populations (Calow 1991). Further measurements of energy resources and comparison of cocoon shapes and diameters would help verifying this hypothesis.

Impairment of earthworm growth at juvenile stage by contaminants was showed in *A. caliginosa* exposed to organophosphate pesticides, whereas adult weights weren't affected(Booth and Halloran ,2001). Also, Mosleh *et al* (2003) evidenced weight losses in mature *A. caliginosa* and *Lumbricus terrestris,* induced by several pesticides applied to artificial soil. In the present study, *A caliginosa* juvenile growth was reduced in glyphosate contaminated soil and in particular in the glyphosate single treatment (and not in mixed with fungicide), but the high individual variations prevented significance of the results. The tendency observed in the exposure scenario with environmentally realistic application rates (low concentrations) is very likely to turn into significant growth reduction over longer time of experimental exposure or within the context of additional stressors in the field.

The present study adds to the knowledge that long-term exposure to pesticides in agricultural soils leads to a reduction in fecundity as measured by cocoon hatching rate, even when the worms are not exposed (i.e. cultured in clean soils).

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